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THE BIOLOGICAL BULLETIN

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EFFECT OF UREA, THIOUREA, PHENYLTHIOUREA AND THIOURACIL ON THE OXYGEN CONSUMPTION OF BLOCKED AND ACTIVE EMBRYONIC CELLS¹

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INTRODUCTION

Considerable data on the action of urea, thiourea, thiouracil and related compounds on the intact organism are at hand, especially as regards their action in such phenomena as growth and thyroid function (Lynn, 1948). That thiourea and thiouracil are important in the activity of the thyroid gland has been widely demonstrated both clinically and otherwise. The exact mechanism of their action on cells, however, seems to be less well understood. The fact that certain oxidations, involving cytochrome-cytochrome oxidase or the peroxidase systems, are significant in the reactions of thiourea and thiouracil seems well established (Sadhu, 1948). It becomes of some interest, therefore, to compare the effects of such chemicals upon the respiratory mechanisms of embryonic cells, both in the mitotically active as well as in the resting or blocked condition. The present paper has to do with results of studies on the effects of urea, thiourea, phenylthiourea and thiouracil upon the oxygen uptake of mitotically active and blocked cells of the embryo of the grasshopper, *Melanoplus differentialis*.

MATERIALS AND METHODS

Eggs of the grasshopper, *Melanoplus differentialis*, were obtained and dissection of embryos carried out as previously noted (Bodine and Boell, 1934). Sterile phosphate-buffered Ringer solution (pH 6.8) was used as the suspending medium for the embryos. All solutions of chemicals (c.p.—urée, thiourea, phenylthiourea, thiouracil²) were made up in this buffered Ringer solution. Diapause (blocked) and post-diapause (active) embryos were, from all external appearances, morphologically identical (Bodine and Boell, 1936). Only embryos from eggs

¹ Aided by grant from the National Institute of Health.

² Samples of thiouracil were generously donated by the Lederle Laboratory of Pearl River, N. Y.

of known temperature and developmental histories were employed. Diapause eggs were those kept constantly at 25° C. from the time of laying, and which were then confirmed by O₂ uptake determination to be in the blocked state. Post-diapause eggs were those diapause eggs previously kept at 5° C. for periods long enough to remove the block and which were also confirmed by further O₂ consumption tests to be in the active state.

Oxygen determinations were carried out at 25° C. with standard Warburg equipment, using 5 cc. flasks. Control or blank runs on all embryos were for 30 minutes, after which the reagents were added from the side arms and the oxygen consumption rates followed for periods of no less than one hour. Three banks of manometers, 18 in number, were used in individual runs and general averages calculated. Each concentration of reagent was tested many times, so that over-all reading totals for each point represent several hundred determinations. One hundred embryos were used in each flask. In comparing reactions of blocked and active embryos, all determinations on a single lot were carried out simultaneously, so that experimental conditions were kept similar throughout.

Concentrations of reagents were calculated from the quantities added to the vessels and it was found that the degree of effect shown by 50 embryos compared favorably with that for 100 embryos, indicating that no appreciable amounts of the reagents were taken up by the embryos.

RESULTS

Urea

The results of the effects of urea upon the oxygen uptake of both active and blocked cells of embryos are graphically depicted in Figure 1. An inspection of

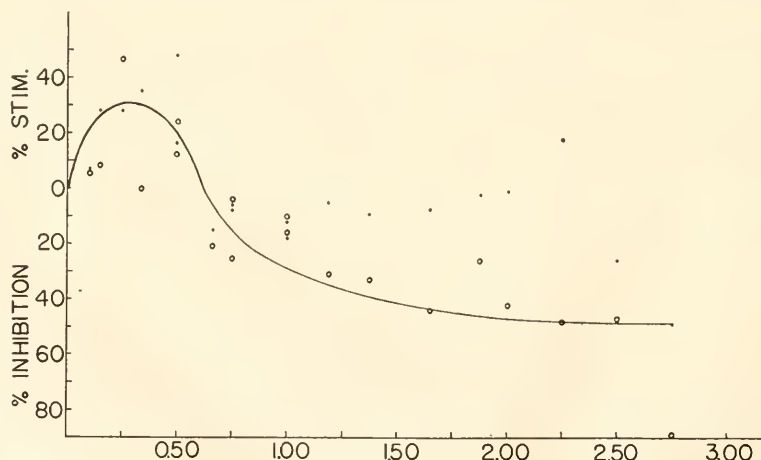


FIGURE 1. Effect of concentration of urea on the percentage stimulation or inhibition of the oxygen consumption of blocked and active embryos. Ordinate, percentage stimulation (top) or inhibition (bottom). Abscissa, molar concentration of urea. Solid curve is average for all experiments on blocked and active embryos for low concentrations and for active embryos in high concentrations. Results for a typical experiment are indicated—solid circles for blocked, open circles for active embryos.

this figure shows a marked stimulation of the oxygen consumption with low doses of urea and a corresponding inhibition with higher doses. The general shape of the curve is similar to that found for ethyl carbamate (Bodine and Fitzgerald, 1948), and is perhaps more or less typical of the reaction of this material to many such reagents. No significant differences in the response of blocked and active cells are apparent for the stimulating effects of low concentrations of urea. For higher, inhibitory doses, however, a marked resistance to the reagent is shown by the blocked cells, the mitotically active cells being much more affected by such exposures. Up to concentrations of approximately 2 molar, the inhibitory effects of the urea on the oxygen uptake of blocked cells are small and completely reversible. For the active cells at concentrations of 1.25 molar, one gets about 50 per cent recovery in oxygen consumption rates, while at 2.5 molar the maximum average recovery amounts to but 25 per cent.

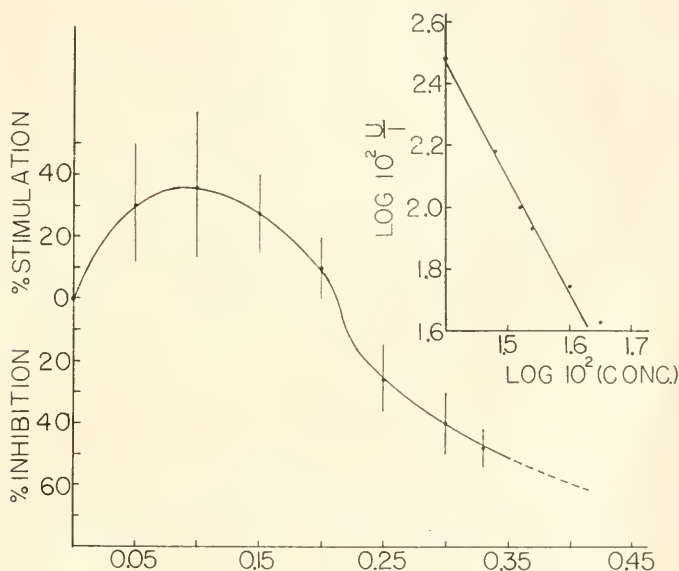


FIGURE 2. Lower left, effect of concentration of thiourea on the percentage stimulation or inhibition of the oxygen consumption of blocked and active embryos. Ordinate, percentage stimulation (top) or inhibition (bottom). Abscissa, molar concentration of thiourea. Solid curve is average for all experiments. Vertical lines through points represent extent of variation for specific concentrations. Upper right, a log-log plot showing relation between ratio of uninhibited (U) and inhibited (I) respiration and concentration of thiourea. Ordinate, $\log 10^2 U/I$. Abscissa, $\log 10^2$ concentration of thiourea.

A point of some interest as regards the reaction of the embryos, both blocked and active, to urea is that after exposure to the reagent and subsequent washing and suspension in Ringer solution, a decided swelling is observed. The intensity of this swelling seems dependent upon the concentration of urea employed. Such a reaction has been observed only after exposure to urea and no other compound. Further details concerning this reaction will be dealt with in a subsequent report.

Thiourea

The effects of thiourea on the oxygen consumption of cells are qualitatively similar to those found for urea. Results are shown graphically in Figure 2. Marked stimulation of oxygen uptake by low concentrations and inhibition by higher concentrations are invariably produced. No significant differences in response to any concentration of the thiourea by the blocked and active cells are noted. Practically complete recovery in oxygen uptake occurs, except for the highest concentrations of reagent employed. The relative effective concentrations of thiourea, as will be noted from Figure 2, are much lower than for urea.

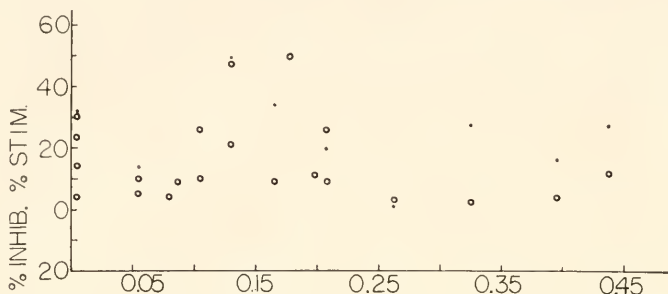


FIGURE 3. Effect of concentration of phenylthiourea on the percentage stimulation or inhibition of the oxygen consumption of blocked and active embryos. Ordinate, percentage stimulation (top) or inhibition (bottom). Abscissa, molar concentration $\times 10^2$ of phenylthiourea. Results for typical experiments indicated. Solid circles for blocked, open circles for active embryos.

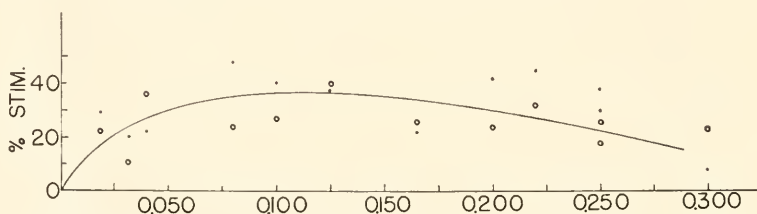


FIGURE 4. Same as Figure 3, but for thiouracil. Solid curve is average for all experiments. Results for typical experiment shown.

Phenylthiourea

The action of phenylthiourea on the oxygen uptake of both blocked and active cells is quite similar to that for urea and thiourea. As indicated in Figure 3, only stimulation of the oxygen uptake has been found for the concentrations employed. Such a result is doubtless due to its low solubility and to the low concentration of the compound used. Complete recovery is found for all concentrations of the reagent.

Thiouracil

Thiouracil, like phenylthiourea, is but slightly soluble and hence only low concentrations of this reagent are available. A marked increase in oxygen uptake

for both blocked and active cells is found (Fig. 4). No significant differences in response for blocked or active cells are noted. When washed and resuspended in Ringer solution, complete recovery in oxygen uptake occurs.

DISCUSSION OF RESULTS

The rather marked and similar effects of urea, thiourea, phenylthiourea and thiouracil upon the oxygen uptake of both blocked and active embryonic cells are indeed striking, and several points of general interest in respect to their fundamental action arise. The fact that thiourea and thiouracil have been shown to be involved in the respiratory mechanisms of cells (Sadhu, 1948) would lead one to suspect similar actions on the oxygen uptake mechanisms of the type of cells employed in these investigations.

The oxygen consumption rates of blocked and active embryos (those mitotically blocked or active) are markedly different (Bodine and Boell, 1934). For morphologically similar embryos, the rates of oxygen uptake of active embryos are at least three to four times that of blocked ones. It seems important, therefore, in any discussion of the effects of various reagents upon the oxygen consumption of these forms, to keep in mind these basic differences in rates of oxygen uptake associated with their cellular behavior. Urea is the only compound found to differentially affect the oxygen consumption of the embryos, and then in such high concentrations as to be rather toxic for the active ones. Blocked cells are but little affected by relatively high concentrations of urea, and then in a completely reversible manner. For all other compounds employed, no significant differences in the response of blocked and active cells are found. Any consideration of the effects of these compounds, therefore, will refer equally to both blocked and active cells.

The characteristic curve of response to different concentrations of these reagents—a marked stimulation of oxygen uptake in low concentrations and a similarly marked inhibition in higher concentrations—compares favorably with results found for ethyl carbamate (urethane) and related compounds (Bodine and Fitzgerald, 1948). Urethanes are thought to have marked effects upon the dehydrogenases or carriers of the respiratory mechanisms of cells. Thiourea and thiouracil inhibit certain enzyme systems, probably the oxidase and peroxidase activity of cells (Sadhu, 1948).

Many invertebrates, as is well known, use copper in functions normally taken over in higher forms by iron, for example haemocyanin. Grasshopper embryos, at the stages used in the present work, contain appreciable amounts of copper (approximately 0.025 μ gm. per embryo³), and it is reasonable to assume that it functions for them in enzyme systems in a manner comparable to other cations for higher forms. It is also well known that copper has marked action upon many enzyme systems, and especially sulfhydryl-containing enzymes (Barron and Singer, 1945). As a specific example, it has been rather clearly shown that copper markedly inhibits the hemolysis of red blood cells in isosmotic glycerol solutions (Jacobs and Carson, 1934). Explanations of such phenomena suggest the action of copper on a sulfhydryl-containing enzyme system located in the red blood cell (LeFevre, 1948). It becomes of some interest, therefore, to examine the possibility of the part played by the contained copper of grasshopper embryos in their reactions to the reagents employed.

³ Unpublished data from this laboratory.

It can readily be shown that tyrosinase, a copper-containing enzyme, isolated from the grasshopper egg, is markedly inhibited by thiourea.⁴ Such a reaction for this enzyme is well known, and the explanations based upon a copper-thiourea reaction seem well founded (Denny, 1943). The possibility of such copper combinations with the other reagents employed seems worthy of comment.

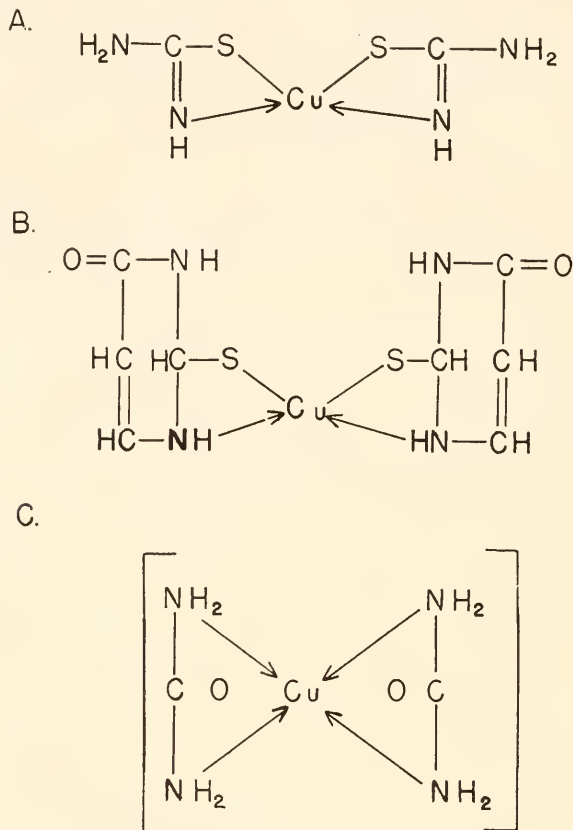


FIGURE 5. Scheme for possible combinations of reagents with copper. A = thiourea, B = thiouracil, C = urea.

For thiourea and thiouracil (A and B of Fig. 5⁵), there is some evidence for the existence of the copper combination as indicated. Theoretically, there is no reason for which a complex copper-urea ion could not exist for urea (C of Fig. 5) in a form essentially as shown. Recently it has been pointed out by LeFevre (1948) that the molecular concentration of reactivating substances (containing SH groups) had to equal, or exceed by one to two times, the concentration of the inhibitor (copper) used in the case of hemolysis of red blood cells. Experiments carried out in

⁴ Unpublished data from this laboratory.

⁵ Acknowledgment is made to Professors George Glockler and Ralph L. Schrinier of the Department of Chemistry for their suggestions in this problem.

connection with the present investigations, using concentration of copper and of thiourea in such proportions, corroborate the findings of LeFevre.

The effects of thiourea and thiouracil on cell respiration and cell division have been variously reported; in some cases inhibition, while in others stimulating responses, are described (Fearon, 1942). Embryos of the grasshopper while in the blocked condition have been shown to lack mitotic activity and to have rates of oxygen uptake at a low constant level. When active, mitosis is always present and rates of oxygen uptake are increasingly higher than for the blocked or inactive states. Such naturally-occurring cellular conditions, therefore, make possible checks on the parts they play in reactions to various reagents. Urea is the only compound with which a marked and significant difference in response between blocked and active cells is noted. Blocked cells are extremely resistant to this compound while active ones are markedly affected, and usually in an irreversible manner. No explanation for such a basic difference in response is at hand.

Published data concerning the effects of urea, etc., on the respiration of cells are fragmentary or almost completely lacking, so that comparisons between copper-containing cells like those used in the present experiments, and those for higher forms, seem practically impossible. The bacteriostatic action of urea alone and in combinations with the sulfonamides seems well established (Kirby, 1943). Alkyl ureas have been shown to depress certain respiratory enzymes (dehydrogenases, coenzymes) (Grant and Krantz, 1942). The effects of thiourea as well as of thiouracil upon such basic phenomena as mitosis, however, seem at present rather confused (Paschkis, Cantarow, Rakoff and Rothenberg, 1945). The effects of thiourea and thiouracil upon the growth of amphibians, as well as upon the thyroid gland itself, are rather clear and well defined (Lynn, 1948).

The stimulating effects of low concentrations of all reagents, although typical, are for the present not readily explained. Permeability and other factors have been suggested for similar phenomena in other forms (Lillie, 1916). Lack of inhibition of oxygen uptake in the case of phenylthiourea and thiouracil appears to be the result of the low solubility of these compounds, and thus for them solubility becomes the limiting factor.

The most regular and consistent responses for any reagent used are those for thiourea, and even here solubility of the compound becomes the limiting factor in a complete analysis of its effect on oxygen consumption. Responses to it for blocked and active cells are not significantly different. An analysis of its inhibitory effects according to the law of mass action, as pointed out by Fisher and others (Fisher and Henry, 1943), would suggest that its action may be similar to that for urethane and that a single respiratory mechanism may be involved (Fig. 2).

SUMMARY AND CONCLUSIONS

1. The action of urea, thiourea, phenylthiourea and thiouracil on the oxygen uptake of the blocked and active cells of the embryo of the grasshopper, *Melanoplus differentialis*, has been studied.

2. In general, low concentrations of these reagents produce stimulation, while higher concentrations produce inhibition of the oxygen consumption of both blocked and active cells.

3. Urea alone produces a differential effect, in that blocked embryos are little affected by high concentrations while active embryos are irreversibly inhibited.

4. The relative effectiveness of the compounds upon the oxygen consumption of blocked and active cells is: thiouracil > phenylthiourea > thiourea > urea; recovery seems to work in the opposite order.

5. Suggestions are made as to the possible significance of copper in determining the basic reaction of these compounds.

ACKNOWLEDGMENT

Acknowledgment is gratefully made to Etta Andrews, John Johnston, and Herman Tharp for technical assistance in carrying out these experiments.

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THE EFFECTS OF ELECTROLYTES AND SUGARS ON THE ERYTHROCYTES OF THE TURTLE, *CHELYDRA* *SERPENTINA*¹

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The erythrocytes of the snapping turtle, *Chelydra serpentina*, exhibit a particular sensitivity to the lack of calcium and will hemolyze in electrolyte solutions which do not contain this ion, though not in pure glucose (Lyman, 1945). The absence of hemolysis reported for glucose is presumed to be due to impermeability to the glucose molecule because of its size. If this is true, sugars of smaller molecular volume might prove hemolytic. Two factors other than size may also have an influence. From Gibbs-Donnan equilibrium relationships, changes in membrane structure may be expected when a non-electrolyte replaces an electrolyte medium, all sugars having an equivalent action in this respect. In addition, sugars might affect the cell membrane by direct chemical action, and such effects may be characteristic for the individual sugars. With regard to the hemolytic action of calcium-free electrolyte solutions on *Chelydra* erythrocytes, it is not known to what extent electrolytes differ in their action, nor in what manner an alteration of the hydrogen ion concentration of any given solution may influence the hemolytic process. These various factors have been considered in the present study.

The results show that hemolysis occurring in calcium-free electrolyte solutions is influenced by the ionic composition of the medium. Moreover, by suitable adjustment of the hydrogen ion concentration, the integrity of the cell membrane may be maintained for short periods even in the absence of calcium. In an examination of the effects of isosmotic solutions of various sugars, striking differences were found, some being hemolytic and others not. Agglutination and hemolytic reactions observed in certain sugars indicate that these compounds are not inert, but produce a definite alteration of the cell membrane.

MATERIAL AND METHODS

Blood (0.5 — 1.0 ml.) of *C. serpentina* drawn without anticoagulant was washed twice in 40 ml. of frog Ringer and suspended in Ringer. In determining the rate of hemolysis, washed cells were centrifuged briefly; the supernate was very carefully removed, and 5.0 ml. of experimental solution added to give a cell concentration of approximately 1:100. The optical density was measured within the first minute and at intervals with a Fisher electrophotometer (Wilbur and Collier, 1943). A Beckman spectrophotometer, made available through the kindness of Dr. W. J. Dann, was used for determinations of the optical density of hemoglobin solutions and for the hemoglobin spectrum of turtle blood.

¹ Aided by a grant from the Duke University Research Council.

Hematocrit tests showed frog Ringer to be approximately isotonic with slight variations between individuals. Experimental solutions were made isosmotic with 0.125 molal NaCl. Double distilled water was used throughout. Galactose, xylose and arabinose were Pfanstiehl brand and were free of calcium. NaCl was Merck Reagent For Biological Work. Other chemicals were reagent grade and were not further purified.

We wish to thank Dr. M. H. Jacobs and Dr. H. B. Collier for their helpful suggestions and Mr. N. G. Anderson and Mr. R. L. Rigsbee for photographic work.

RESULTS

Electrolytes

The observation of Lyman (1945) that erythrocytes of *C. serpentina* will hemolyze in isotonic Ca-free salt solutions was readily confirmed. Moreover the course of hemolysis in isosmotic NaCl, for example, could be arrested by the addition of a small amount of isosmotic CaCl_2 solution to the hemolyzing suspension. However, in preliminary experiments it became apparent that the rate of hemolysis varies with the cation and anion employed and also with the hydrogen ion concentration.

The effect of different cations was examined by following the course of hemolysis in buffered and unbuffered isosmotic solutions of NaCl, KCl, MgCl_2 and CaCl_2 . Hemolysis was always most rapid in KCl, followed by NaCl, slower in MgCl_2 (Figs. 1 and 2), and completely absent in CaCl_2 .

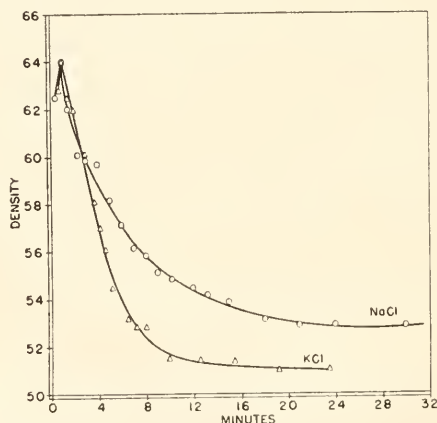


FIGURE 1. Hemolysis of *Chelydra* erythrocytes in 0.125 molal NaCl and 0.126 molal KCl. 29° C. pH 7.41. Solutions buffered with corresponding isosmotic phosphate in the proportion 9:1. The initial rise in the curves indicates preliminary shrinkage probably due to slight hypertonicity. The final values for optical density represent complete hemolysis for both solutions, the difference apparently resulting from difference in opacity of the ghosts.

The rate of hemolysis in any given electrolyte solution was always greater in an alkaline than in an acid solution. This could be shown by adjusting the pH with HCl or the hydroxide of the cation being studied, or by use of phosphate buffers. Figure 3 illustrates the effect in the case of KCl buffered with isosmotic phosphate.

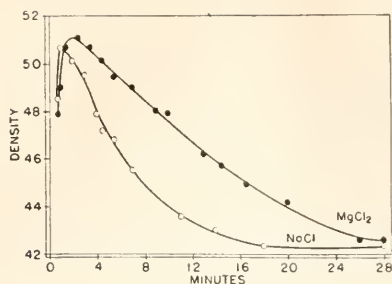


FIGURE 2. Hemolysis of *Chelydra* erythrocytes in 0.125 molal NaCl and 0.088 molal MgCl_2 . 28° C. pH 7.41. Solutions buffered with 0.1 molal sodium phosphate 9:1.

NaCl behaved similarly. The rate showed little change within the range pH 7.8 to pH 6.7 but was definitely decreased at pH 5.9 to pH 6.1. The inhibitory effect of acidity was demonstrated in another manner (Fig. 4). Cells were placed in NaCl buffered to pH 7.0 with a trace of phosphate. After hemolysis was well under way acidified NaCl was added. Hemolysis was quickly arrested. On the addition of alkaline NaCl hemolysis was resumed at the original rate, indicating reversibility of the inhibition. If cells remain in the acid NaCl longer than about 10 minutes, hemolysis will be resumed at a very slow rate. When the pH is restored to the alkaline range after 50 minutes the former rapid rate is regained.

The effects of the cyanide and citrate of sodium and potassium were compared with the corresponding chloride at pH 7.25 or 7.4. Isosmotic mixtures of chloride and cyanide containing 0.019 molal cyanide exhibited a hemolytic action similar to that of isosmotic chloride. The same results were obtained with cells exposed to

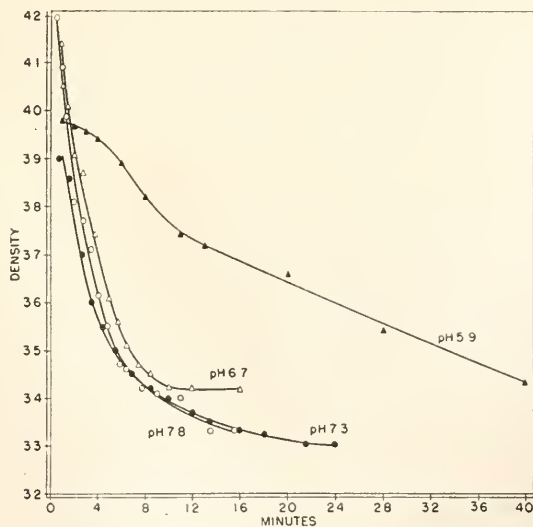


FIGURE 3. Hemolysis of *Chelydra* erythrocytes in 0.126 molal KCl buffered with 0.1 molal potassium phosphate 9:1. 28° C.

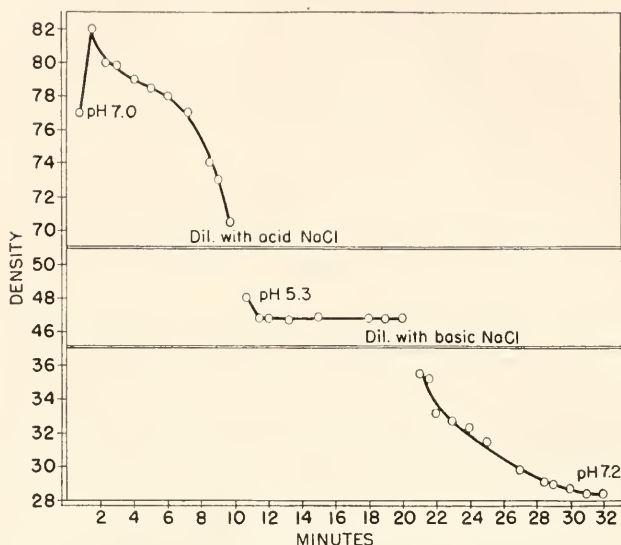


FIGURE 4. Effect of acidity on hemolysis of *Chelydra* erythrocytes in 0.125 molal NaCl. Ten minutes after the erythrocytes were mixed with 4.0 ml. NaCl solution (pH 7.0), and during the course of rapid hemolysis, 3.0 ml. of acid NaCl was added bringing the pH to 5.3. Hemolysis was arrested within one minute. Addition of 1.9 ml. of alkaline NaCl at 20 minutes raised the pH and hemolysis continued immediately. Dilution gave a marked change in optical density as indicated. The initial rise in the curve indicates shrinkage probably due to hypertonicity. 21° C.

the same concentration of cyanide in Ringer for 30 minutes before adding the hemolytic solution. The absence of any marked effect is not surprising in view of previous work with other electrolytes (Davson and Danielli, 1938; Hunter, 1947). Isosmotic sodium citrate gave slower hemolysis than NaCl, which may be the result of impermeability to the citrate ion as compared with chloride (Jacobs, 1940).

Sugars

When *Chelydra* erythrocytes are suspended in unbuffered isosmotic dextrose solution, agglutination occurs followed by hemolysis. The amount of hemolysis can be determined by removing the cells by centrifugation and measuring the concentration of hemoglobin in the solution. If the suspension is pipetted up and down, the masses of cells tend to break up and hemolysis is further increased. In dextrose buffered at pH 7.5 with a small amount of sodium phosphate, agglutination was no longer apparent; and, as might be expected from the effect of pH on hemolysis in salt solutions, hemolysis was more pronounced.² A comparison of the hemolytic action of various sugars, including one disaccharide, two hexoses, and two pentoses (Table I), showed that the pentoses differed markedly from the other sugars, hemolysis being absent in arabinose and very slight in xylose. Dextrose gave complete hemolysis. On centrifugation of such hemolyzing suspensions, a

² The buffer may well have an effect as an electrolyte.

jelly-like mass containing nuclei, many distorted and a few normal erythrocytes, was found. This contrasts with hemolysis in electrolytes in which normal ghosts were present, with about 50 per cent of the ghosts spherizing in isosmotic MgCl_2 .

Observations on agglutination indicate that differences exist between sugars in this respect as well as in their hemolytic action. Cells (0.07–0.08 ml.) suspended in Ringer were centrifuged briefly; the supernate was carefully removed and 8.5 ml. of isosmotic sugar solution was added. After stirring to give uniform cell distribution, the suspension was left undisturbed.³ Results were similar for three individuals (Table I). While strict comparisons between certain sugars cannot be made because of differences in pH, it is apparent that all sugars cause agglutination, but all are not equivalent. Moreover, the effects of agglutination do not parallel hemolytic actions. So, for example, sucrose is hemolytic and weakly agglutinating; arabinose, on the other hand, has a greater agglutinating action without producing hemolysis.

TABLE I

Hemolysis and agglutination in isosmotic sugar solutions

Cells were removed from solutions after 3 hours and the optical density of the supernate was measured at 576 millimicrons.* pH 7.5–25° C. Blood from three turtles.

	Hemolysis		Agglutination	
	Sample 1	Sample 2		pH
dextrose	0.630	0.510	++++	5.4
sucrose	0.344	0.378	+	5.7
d-galactose	0.325	0.163	+	4.5
d-xylose	0.087	0.017	++++	4.4
l-arabinose	0.007	0.005	+++	5.7
Ringer	0.017	0.014	0	5.7

* After initial mixing the suspensions were left undisturbed to minimize hemolysis and then centrifuged. Centrifugation may increase the hemolysis slightly. (Collier, 1948. Personal communication.) The absorption spectrum was determined for one sample of blood, and maxima were located at 543 and 576 millimicrons. These agree closely with values given for human blood.

The influence of electrolytes on agglutination may be shown by adding one part of isosmotic NaCl , KCl , MgCl_2 or CaCl_2 to 14 parts of isosmotic dextrose. Agglutination was inhibited somewhat by the addition of electrolytes in all cases and was less pronounced in the presence of NaCl and MgCl_2 than with the other salts (Fig. 5). But in no instance was agglutination completely prevented. Microscopic observations indicate similar differences (Figs. 6 and 7). Quantitative aspects of this effect, previously studied on other cells (Radsma, 1918), have not been investigated.

³ Sedimentation rate fails to give a measure of the extent of agglutination in this blood inasmuch as some cells do not agglutinate and therefore sediment relatively slowly, whereas the agglutinated masses in the same suspension fall rapidly or adhere to the wall of the tube. Adherence to the wall is observed in vertical tubes of 1.0 cm. or more in diameter.

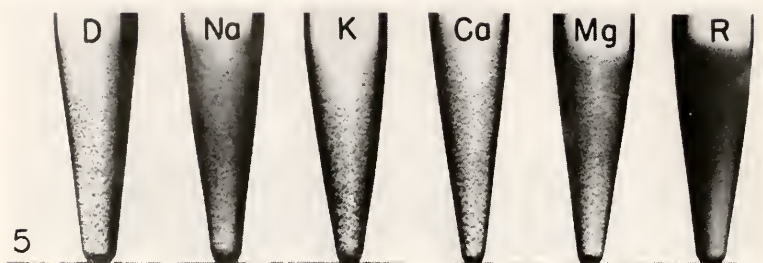


FIGURE 5.

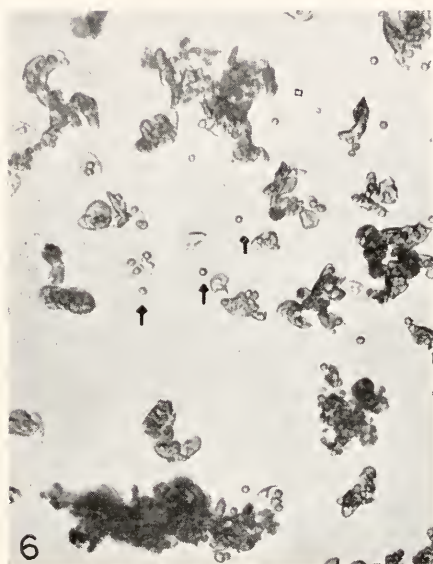


FIGURE 6.



FIGURE 7.

FIGURE 5. Effect of salts on agglutination of washed *Chelydra* erythrocytes in dextrose solution. Isosmotic salt solutions were added to unbuffered dextrose (0.23 molal) in the proportion 1:14. D—dextrose; Na—NaCl + dextrose; K—KCl + dextrose; Ca—CaCl₂ + dextrose; Mg—MgCl₂ + dextrose; R—Ringer (unbuffered). After initial mixing tubes were left undisturbed. 26.5° C.

FIGURE 6. *Chelydra* erythrocytes in 0.23 molal unbuffered dextrose. Washed cells were mixed with dextrose solution and placed immediately on a slide without coverslip. Arrows indicate ghosts.

FIGURE 7. *Chelydra* erythrocytes in 0.23 molal unbuffered dextrose with isosmotic NaCl 14:1. Shrinkage may be noted.

DISCUSSION

Consideration of the mode of action of electrolytes and non-electrolytes on *Chelydra* erythrocytes is complicated by the fact that related substances may be strikingly different in their actions. Thus, no single scheme will serve to explain completely the action of sugars, nor can cation effects be interpreted adequately in

terms of valence. The latter is in contrast to human erythrocytes, in which ions of the same valence are alike in preventing loss of salts from cells in sucrose solution (Wilbrandt, 1940).

The effect of calcium in maintaining the normal permeability characteristics of the cell may be considered in relation to (1) the thickness of the ionic double layer and the adhesion of membrane components, and (2) crossbinding of anions within the membrane (Danielli, 1937, 1943). In both respects calcium has a more pronounced action than sodium or potassium. Following the Gibbs-Donnan equilibrium, with a change of medium from Ringer to isosmotic NaCl or KCl as was done in the present experiments, there will be a replacement of calcium in the surface layer. Compactness of the membrane will be decreased, which may in turn lead to an increase in permeability resulting in swelling and hemolysis. Such an increase in volume preceding hemolysis in these solutions may be readily observed under the microscope. Additional assumptions will be necessary to explain such differences as those found between the effects of calcium and magnesium on hemolysis and on sphering of ghost cells.

With sugar solution as the medium, the salt concentration essentially zero, and a constant anion concentration in the cell surface, there is to be expected from the Gibbs-Donnan equilibrium a decrease in membrane concentration of mono- and divalent metal ions, and an increase in surface acidity, which has been thought to be of sufficient magnitude to alter the proteins of the membrane and accordingly cell permeability (Danielli, 1937; Wilbrandt, 1940). However, in this cell an increase in acidity of the medium stabilizes the membrane, as shown by the acid inhibition of hemolysis in both electrolytes and non-electrolytes. At the same time the loss of metal ions would result in increased repulsive forces within the membrane, giving greater distances between molecules and an increase in permeability (Danielli, 1943). Even though it is assumed that the net effect favors an increased permeability, the present results are not completely explained inasmuch as all sugars should behave similarly, whereas some have been shown to be hemolytic while others are not.

Other factors to be considered are a differential permeability to sugars and effects of individual sugars on membrane structure. Any differences in permeability which may exist must involve factors other than molecular volume since this does not correlate with hemolytic action. So, for example, sugars which were least hemolytic (pentoses) have the smallest molecular volume. (See also Ulrich, 1934.) The agglutination of cells indicates an alteration of the cell surface by the sugar, the degree to which this occurs depending upon the particular sugar and ionic composition of the medium. Further, hemolysis in sugar, contrary to the results in electrolyte solutions, was characterized by disintegration of many of the cells, again pointing to a direct action on membrane structure.

The effect of acidity in decreasing hemolysis obtained with electrolyte solutions suggests that molecular rearrangements within the membrane may in part compensate for the lack of calcium. The fact that the erythrocyte is stable in certain Ca-free non-electrolytes indicates that it is not the absence of calcium *per se* which causes hemolysis in electrolytes, but rather the effect of other cations which may replace the calcium of the cell membrane and so increase its permeability. The cell, then, would be sensitive to lack of calcium only because of the ready replacement of its calcium by other cations.

SUMMARY

1. The comparative hemolytic rates of *Chelydra serpentina* erythrocytes in isosmotic salt solutions as measured photometrically were. $KCl > NaCl > MgCl_2$, and $NaCl > Na_3$ citrate. Hemolysis in cyanide (0.019 molal) was similar to that in chloride. No hemolysis occurred in isosmotic $CaCl_2$ and the addition of $CaCl_2$ to cells hemolyzing in Ca-free electrolyte solutions arrested hemolysis at once.

2. Hemolysis in sodium and potassium solutions was greatly retarded at about pH 6 and below.

3. The hemolytic potency of isosmotic sugar solutions (pH 7.4) was found to be: dextrose $>$ sucrose $>$ d-galactose $>$ d-xylose with complete hemolysis in dextrose and none in l-arabinose in three hours.

4. Sugar hemolysis was accompanied by abnormal shape changes and disintegration of cells, whereas in Ca-free electrolyte solutions "normal" ghosts were found.

5. Agglutination occurred in unbuffered isosmotic sugar solutions, the extent depending upon the particular sugar. Agglutinating action was not correlated with hemolytic potency.

6. Results of experiments on the hemolytic and agglutinating properties of sugars indicate that certain sugars are not inert but have a definite action on the cell surface.

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EXPERIMENTS ON THE DETERMINATION AND DIFFERENTIATION OF SEX IN THE BOPYRID STEGOPHRYXUS
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One of the great controversies related to the Bopyridae (a family of isopod crustaceans, suborder Epicaridea), and one that has broader biological implications as well, is the question of sex-determination. Is sex already determined in the larval stage, or does the fate of the larva (i.e. whether it turns into a female or a male) depend on environmental influences? Can such external factors as the position the larva occupies or the nourishment it receives bring about sex reversal in an already sex-determined larva?

The chief views expressed in the past regarding this question are as follows:

1. Giard and Bonnier (1887) maintained that in the Bopyridae all free-swimming larvae are males. The first larva, however, that invades a particular host undergoes sex reversal and transforms into a large female without ever having functioned as a male. The next to come settles on this female and metamorphoses into an adult dwarf male which fertilizes the former.

2. Smith (1909) and Goldschmidt (1920) stated that all the species of Epicaridea, Bopyrina as well as Cryptoniscina, are protandric hermaphrodites, each individual being male while in a larval state, and then losing its male organization and becoming female as the parasitic habit is assumed. The females, therefore, result from males that have already functioned as males.

3. Hiraiwa (1936) believed that the free-swimming larvae are not males but are sexually undifferentiated, although the sex is already predetermined. Differentiation follows fixation, but is probably not due to environmental factors.

4. Recently, Caullery (1941), impressed by the influence of association on sexuality as exhibited in such animals as *Bonellia*, *Crepidula*, and *Ophyotrocha*, made the suggestion that the sexes may not be fixed from the start, but that direct parasitism of a larva on a host entails differentiation into a female, and indirect parasitism, through the intermediary of a female on which it is stationed, entails differentiation into a male. Lacking direct evidence, however, he suggested an experimental approach to test the validity of this theory. He advised collecting the newly-arrived cryptoniscid larvae that can frequently be found in the brood pouch of a female bopyrid—larvae which according to this view would evolve into typical males under the influence of the environment—and placing them in contact with young crabs not yet parasitized. Caullery thought it probable that these larvae would fix to the crab and become females.

These suggestions of Caullery moved the writer to undertake a series of experiments with the larvae of *Stegophryxus hyptius* Thompson, an ectoparasite of the

¹ Supported in part by a grant from The Catholic University of America Research Fund.

hermit crab *Pagurus longicarpus* Say. This work was carried on in the summer of 1946 at the Marine Biological Laboratory, Woods Hole, Mass. In addition to the experiment suggested by Caullery, reciprocal experiments were undertaken in which presumptive female larvae were taken from the host crab and transferred to the brood pouch of a female bopyrid to test the suspected masculinizing influence of the female on cryptonisci that attach to her body.

The present paper reporting on this work was ready for publication when the writer's attention was drawn to an article in Italian by Reverberi and Pitotti, which, although it appeared in 1942, had not been mentioned in the abstracting journals until 1947. This paper provides the first experimental verification of any of the proposed sex-determination theories with reference to the Bopyridae. The authors, working with *Ione thoracica* Montagu, showed that the control of sex-determination is environmental rather than genetic. However, since there are several points of difference between the biological cycles and sex phenomena of *Ione* and *Stegophryxus*, it was decided not to alter the present paper as originally written, but in the discussion and footnotes to draw a comparison between the results reported by Reverberi and Pitotti and our own.

LIFE CYCLE OF *STEGOPHRYXUS*

Only about 1.5 per cent of *Pagurus longicarpus* at Woods Hole are parasitized by *Stegophryxus hyptius*. Thompson (1901), in his original description of the species, gave an account of the morphology of the adult female, adult male and some of the immature forms, but the life cycle has heretofore not been discussed.

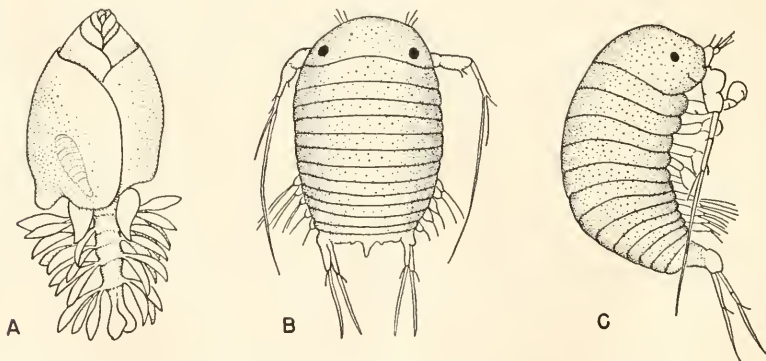


FIGURE 1. Adult female and epicaridium larva of *Stegophryxus hyptius*.

A. Ventral view of adult female. The dwarf male, although not visible externally, is shown within the brood pouch by a dotted outline to indicate its position and relative size. $\times 5$. B. The epicaridium or first larval stage shown in dorsal view. $\times 120$. C. Lateral view of epicaridium larva. $\times 120$.

The female *Stegophryxus*, as is the case in all bopyrids, is much larger than the male (Fig. 1A). It occurs on the abdomen of the hermit crab, to which it is attached, back downward, by its mandibles and legs. Its thorax is concealed ventrally by an enormous brood pouch, made up of five pairs of thin brood plates.

Within this brood pouch lies the slender dwarf male, whose function is not that of inseminating the female and then quitting her, but of remaining in readiness to fertilize the successive batches of eggs that are released into the brood pouch during the female's productive life. These eggs, within two weeks after fertilization, develop into first stage larvae that leave the mother and swim off. After an interval of about five days, the marsupium is again filled with eggs and another brood begins embryonic development.

The first larval stage is known as the *epicaridium*. It is a short, broad, semi-barrel-shaped larva (Fig. 1B) with sub-chelate pereopods for clinging and with pleopods in the form of swimming organs. The epicaridium of *Stegophryxus* measures about $270\ \mu$ in length, $150\ \mu$ in breadth, and $120\ \mu$ in depth (not including the appendages). In this stage the young of *Stegophryxus* escape from the brood pouch and swim off as plankton organisms. In the laboratory, they quickly rise to the surface of the water and remain there floating or swimming about for days.

The subsequent history of the epicarid larva has not been investigated in *Stegophryxus*. It may attach to a pelagic copepod, undergo a molt, and become a *microniscus* larva, which, after feeding on the copepod, will eventually transform into a new larval stage known as the *cryptoniscus* that swims off to seek the definitive host. This type of development is known to occur in some of the Epicaridea (Sars, 1899; Caullery, 1907; Caroli, 1928; Reverberi and Pitotti, 1942). Or the epicarid larva may develop directly into a cryptoniscus larva, an abbreviated type of development which Hiraiwa (1936) believes is the case in most Bopyridae. We postulate the first alternative in the case of *Stegophryxus* because of the great difference in size between its epicaridium and cryptoniscus stages, a difference which can only be accounted for by assuming the existence of an intervening stage.

At any rate, however arrived at, the earliest larval stage of *Stegophryxus* that we find on the crab is the cryptoniscus. In this stage (Fig. 2) the parasite is typically isopod in its characteristics. It has an elongated body, dorsoventrally compressed, segmented and well chitinized. There are seven pairs of thoracic appendages (as compared with six pairs in the epicaridium) all similar in form, six pairs of uniramous natatory pleopods, and one pair of biramous uropods. The cryptoniscus measures about $680\ \mu$ in length, being therefore about two and one-half times longer than the epicaridium.

We have been able to distinguish three phases in the life of the cryptoniscus larva on the basis of color pattern which we shall designate as (1) the brown chromatophore phase, (2) the black chromatophore phase, and (3) the striped contracted phase.

The youngest cryptoniscisci, those that have recently settled on a crab, have a profusion of dark brown expanded chromatophores that cover the dorsal surface of the body in such a way as to leave an uncolored portion that resembles a cross (Fig. 2A). These chromatophores are present laterally on the head and segments 1, 4, 5, 6, 7, and 8; are present centrally as well as laterally on segments 12 and 13 and on the uropods; and are entirely absent from segments 2, 3, 9, 10, and 11. The general body color is pale yellowish and is due to another system of chromatophores, which are scattered over the integument without definite plan. The eyes are reddish brown.

In phase 2, the light colored cross-shaped pattern remains much as before, but most of the areas formerly occupied by brown chromatophores are now occupied

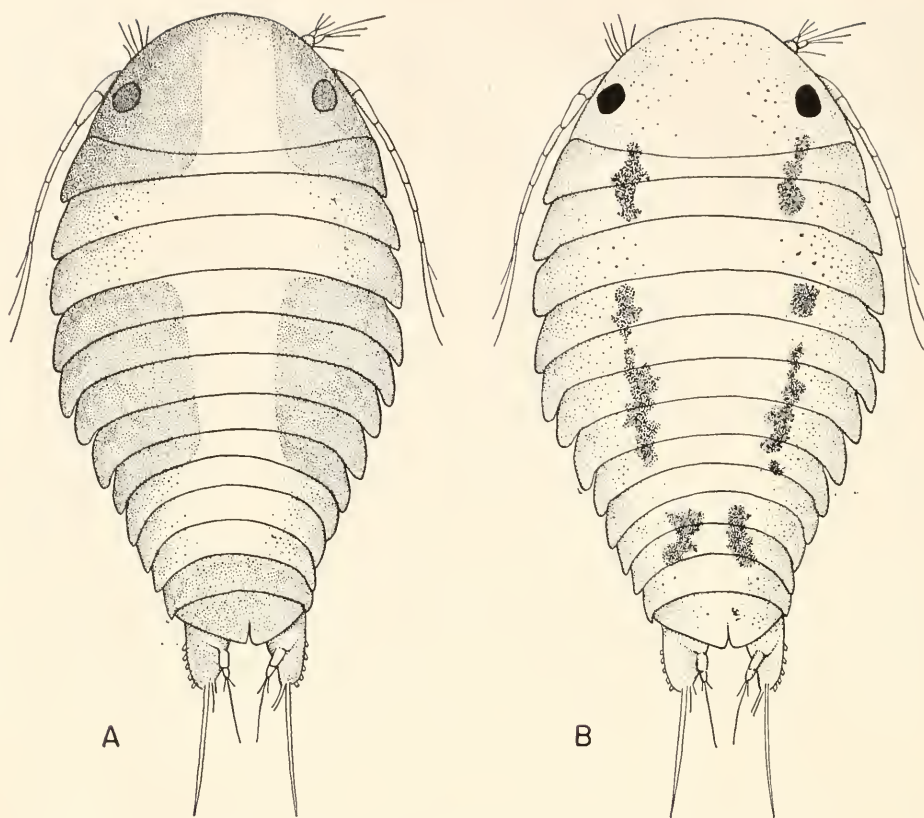


FIGURE 2. The cryptoniscus larva of *Stegophryxus hyptius*.

A. Young larva showing color pattern when in phase 1. The light cross-shaped area is devoid of chromatophores. B. Older cryptoniscus in phase 3 with contracted melanophores forming an interrupted stripe on each side.

by expanded black chromatophores. Their distribution is as follows: present laterally on the head and segments 1, 4, 5, 6, 7, and 8; present centrally on segments 11 and 12; absent from segments 2, 3, 9, 10, 13 and the uropods. The eyes have also become black. The yellow chromatophores are now more noticeable and have become restricted to segments 1 to 11 inclusive where they are present laterally.

In phase 3 (Fig. 2B) the black chromatophores are much fewer in number and are all in the contracted state. They form a broken chain on each side of the body about midway between the center and margin of the dorsal surface, reaching from segment 1 to segment 8 inclusive, but absent on segment 3. On segments 10 and 11 there are a few black chromatophores centrally located. Yellow chromatophores are intermingled with the black in the same chain but extend from segment 1 to segment 11. The eyes are black. In this stage the cryptoniscus is ready for the molt which will transform it into a juvenile female of the first postlarval stage.

No structural differences have been detected in these three cryptoniscid stages. Since neither the brown nor the black chromatophores lose their color in alcohol, they are no doubt melanophores which presumably differ only in the amount of melanin present.

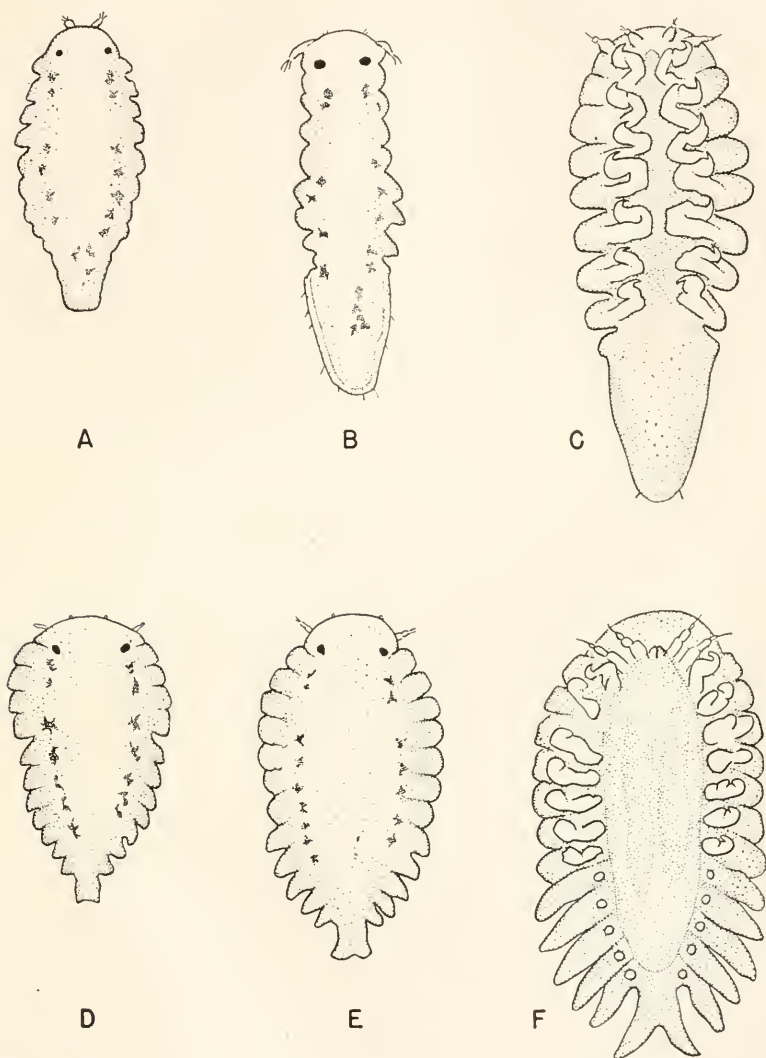


FIGURE 3. Juvenile males of *Stegophryxus hyptius* compared with juvenile females of the same species and same degree of development.

A. Juvenile male in first post-cryptoniscid instar. Specimen drawn measured 0.85 mm. Dorsal view. B. Older juvenile male measuring 1.07 mm. in length. Dorsal view. C. More advanced juvenile male measuring 1.4 mm. in length. Ventral view. D. Juvenile female in first post-cryptoniscid instar. Specimen drawn measured 0.85 mm. Dorsal view. E. Older juvenile female, 1.01 mm. in length. Dorsal view. F. More advanced juvenile female measuring 1.3 mm. in length. Ventral view.

The juvenile female into which the cryptoniscus transforms is shown in Figure 3D. It is broader than the cryptoniscus and the pleon lacks uropods and possesses only stump-like rudiments of pleopods. The terminal segment is extended into a tail-like outgrowth ending in a shallow notch. The animal is whitish with color pattern differing little from that of the last stage cryptoniscus. More advanced juvenile females are shown in Figures 3E and 3F.

About ten per cent of the crabs examined in the summer of 1946 were infested with one or more cryptonisci. This was fortunate, insofar as experimental possibilities were concerned, since infective cryptonisci have rarely been reported. Bonnier, for example, having studied about eighty species, came across cryptoniscus larvae that had recently attached to the host only twice. Hiraiwa never found them during five years' study of *Epipenaeon japonica*. The cryptonisci of *Stegophryxus* may be found on almost any part of the crab, but only those attached to the pleopods have actually settled down. The others are transients or new arrivals that wander off at the slightest disturbance. Even those on the pleopods, while more permanent than the others, are apt to leave when disturbed. The older the cryptoniscus, the more fixed in position it is, and if found attached to the last pleopod (third abdominal appendage of males, fourth of females), the favorite resting site, one can presume that it will remain there, barring accidents, until it eventually transforms into a female. Only after the juvenile female stage is assumed does the parasite leave the pleopod to fix itself permanently on the abdomen proper of the host.

Cryptonisci destined to become functional males are those found attached to a young or mature female. They are identical in form, size, and color pattern with those found on the crab. They too pass through the same three phases, but metamorphose into juvenile males. The first male instar is shown in Figure 3A. It is narrower in form than the corresponding female instar and has a strikingly different type of pleon which is tongue-shaped and rounded at the tip. More advanced juvenile males are shown in Figures 3B and 3C.

TRANSFORMATION OF PRESUMPTIVE FEMALES INTO MALES

The first question to be answered experimentally was whether or not the cryptonisci found on normal crabs, that is on crabs not infested with a female *Stegophryxus*, could be transformed into males. Such larvae in all likelihood would be presumptive females. If removed from the crab and transferred to the brood pouch of a female bopyrid, would these cryptonisci metamorphose into males?

I. In the first series of experiments, cryptonisci taken at random from normal crabs were placed in a dish with a crab parasitized by a mature *Stegophryxus*. The male was first removed from the brood pouch of the female to prevent interference. This was necessary because if the adult male is allowed to remain, although cryptonisci will attach as freely as when no male is present, they will enjoy only a relatively brief period of attachment before they are driven off.

Four experiments of this type were conducted. In all cases the greater proportion of the cryptonisci attached to the female bopyrid and the greater proportion likewise entered the juvenile male phase. But, after varying lengths of residence in the brood pouch and correspondingly varied degrees of attainment of the male phase, all but one out of each lot eventually deserted the female. The one that re-

mained in unchallenged possession eventually became a mature male, and in cases where the experiment was continued long enough this male functioned as such and successfully fertilized the eggs of its consort which then developed normally into epicaridium larvae.

It will be sufficient to cite one experiment of this series in detail. This experiment was begun July 13, 1946 with five cryptonisci taken at random from unparasitized crabs and placed in a dish with a crab having a mature *Stegophryxus* (male removed) whose brood pouch contained late embryos.

July 14. Two cryptonisci have attached to the female *Stegophryxus*.

July 15. Four cryptonisci now present on the bopyrid. Epicarids are hatching.

July 16. Three cryptonisci remain within the now empty brood pouch. They have developed to the black pigment stage.

July 17. Metamorphosis of cryptonisci continuing; one, at least, has molted.

July 19. The three cryptonisci have entered the juvenile male phase and one is slightly more advanced than the others.

July 24. The three juvenile males are still present and continuing their development.

July 26. One of the juvenile males has disappeared. One of the two remaining ones is permanently removed for examination and drawings are made of it.

August 9. The brood pouch of the female bopyrid is now filled with eggs. (This means that the male has reached maturity.)

August 11. The male was removed for measuring and returned to the brood pouch. Its length is 2.28 mm.

August 24. Development of the eggs has continued normally and today the epicarid larvae are released.

August 26. The male now measures 2.37 mm. Experiment discontinued.

Similar results were obtained when female bopyrids, found in nature with a retinue of cryptonisci present in the brood pouch, were kept under observation. In one case a female *Stegophryxus*, non-ovigerous and lacking a male, had 18 cryptonisci attached to it. The daily count showed a reduction as follows: 18, 16, 14, 11, 9, 6, 4, 2, 2, 2, 2, 2, 2, 1. The remaining one reached maturity two weeks later and fertilized the eggs of the female which were not released until that time. Another reduction from an initial natural retinue of eight cryptonisci occurred as follows: from 8 to 2 in four days, but these two persisted for 11 more days to become juvenile males, then one disappeared. The survivor became a mature male.

These experiments and observations, while they shed some light on the problem at hand, are inconclusive evidence for or against any theory of the sexual nature of the cryptonisci. They show that cryptonisci that enter the brood pouch of a female metamorphose in the male direction, but what of those that leave early or fail to enter? Could not they be predetermined females unresponsive to masculinizing influences?

II. To settle this point, it was decided to experiment with single cryptonisci. Moreover, only cryptonisci found clinging to the posterior pleopod of a normal crab were used. Nine experiments were undertaken. In five of these the cryptoniscus selected for insertion in the brood pouch of a female was in the brown chromatophore stage; the four other cryptonisci were in the more advanced black stage.

Each of the five Stage 1 cryptonisci remained in the brood pouch and made no efforts to crawl out. One was removed after six days, one after eight, one after eleven, and two after twelve days. Each one had metamorphosed into a male, whose size and extent of development was proportional to the length of time spent in the brood pouch. Those that had been on the female for eleven or twelve days had reached a size of from 1.5 mm. to 1.8 mm.

The experiments with Stage 2 cryptonisci gave different results. In three cases the cryptoniscus crawled out of the brood pouch within a day or two and was either lost or found clinging to the crab instead. One experiment yielded positive results. This cryptoniscus refused repeatedly to attach to the female, but after each escape it was returned to the brood pouch. Finally it remained there, and, eventually, 28 days later, had become a 2 mm. male.

The positive results obtained with the five Stage 1 cryptonisci strongly indicate that cryptonisci that would ordinarily become females can readily be transformed into males through attachment to the body of the female bopyrid, provided the transfer is effected at an early age. Even the one success with a Stage 2 cryptoniscus confirms this. It must be concluded that after a certain period of parasitism on the crab the cryptoniscus becomes female-determined and the direction of its sex development can no longer be changed under ordinary conditions. Subsequent experiments, using juvenile females for transfer, instead of cryptonisci, support this view and will now be briefly recounted.

III. Four attempts were made to transform juvenile females into males. All were completely unsuccessful. Juvenile females in the early post-cryptoniscid phase were used, before they had developed far enough to leave the pleopod of the host for permanent attachment on the abdomen.

One female remained inside the brood pouch for one day, crawled to the exterior and remained there one day, then disappeared.

One left the brood pouch the day after transfer and attached to the abdomen of the crab where it remained for eight days, when the crab died.

One left the brood pouch on the second day and attached to the underside of the telson of the crab. It remained there until the experiment was discontinued 12 days later, and grew from an initial size of .85 mm. to 1.4 mm.

The fourth one was transferred to the brood pouch together with the pleopod to which it was attached. This female remained for five days, then disappeared and could not be recovered for examination.

It would seem that juvenile females are averse to becoming ectoparasites of other more mature females. They leave such an unnatural situation to return to direct parasitism on the crab.² There is no evidence that any of the four were modified by their brief sojourn in the brood pouch of another female.

² Reverberi (1947) came to the same conclusion with regard to *Ione*. However, he then placed two females together *in vitro* apart from the host, one being a juvenile female and the other an adult from which the juvenile would have to derive its nourishment. As often as the adult died, another of the same age would be substituted. By this ingenious method he was able to maintain a direct association between a juvenile female and an adult *Ione* for several months. One case of definite sexual inversion resulted from many trials of this sort. This particular juvenile female underwent external changes and gradually took on the appearance of a male. When killed and sectioned after nearly four months under these experimental conditions, the individual was found to have normal testes partially filled with sperm.

ATTEMPTS TO TRANSFORM PRESUMPTIVE MALES INTO FEMALES

If presumptive female cryptonisci can be turned into males by altering the environment, the question naturally arises regarding the possibility of producing females from presumptive male cryptonisci. The method of experimentation would be to remove cryptonisci from the brood pouch of a female bopyrid and transfer them to a crab instead. This is the type of experiment on which Caullery pinned his hopes of verifying the theory of sex determination in Epicaridea based upon the type of association with the host.

When this was tried it invariably led to failure because the transferred cryptonisci did not remain attached to the crab long enough to show either positive or negative results. This failure to remain attached need not necessarily be attributed to aversion on the part of the cryptonisci for a strange environment. Indeed, the hazards in the case of direct parasitism on the crab are great. When it is recalled that approximately 10 per cent of the normal crabs have cryptonisci on their surface and only 1.5 per cent of all crabs are infested with female *Stegophryxi*, it becomes clear that many potential parasites are eliminated through environmental difficulties. Moreover, no success was achieved in numerous attempts to rear to the juvenile female stage cryptonisci found naturally attached to crabs. When crabs bearing cryptonisci are isolated in a dish and examined after a day or two, one finds that the cryptonisci have disappeared. Apparently they are eaten by the crab, since cryptonisci kept in dishes without crabs will remain alive for as long as two weeks. It may be remarked in passing that although isolated cryptonisci survive, they do not develop, nor pass from the brown to the black phase. It is significant that the juvenile female *Stegophryxi* naturally occurring on crabs can be reared without difficulty in the laboratory. They are actually fixed to the crab and have lost their ability to swim, whereas the cryptonisci, as explained before, are still active and only perch on the crab without fastening themselves to it. Should they disengage themselves even momentarily, they are in danger of being caught up by the currents passing through the gill chambers of the crab and swept in the direction of the crab's mouth. The mouth parts of the crab are in constant motion and any particle that comes in contact with them is trapped and masticated.

The failure of these experiments with cryptonisci removed from the brood pouch of a female and transferred to a crab may, therefore, with considerable assurance, be laid to experimental difficulties.³ When the proper technique is worked out for *Stegophryxus*, which will eliminate the hazards facing cryptonisci that attach to crabs, we feel confident that presumptive male cryptonisci can be transformed into females.

³ This is especially reasonable in view of the fact that Reverberi and Pitotti (1942) experienced a similar lack of success when they tried to implant the cryptoniscus larvae of *Ione* on the gills of *Callinassa*. The cryptonisci invariably failed to remain on the host (pp. 148-149). But when they used post-cryptoniscid stages they were successful in bringing about the transformation of juvenile males into females. *Ione*, unlike *Stegophryxus*, is a branchial parasite. When juvenile males of *Ione*, removed from adult females, were placed in the branchial cavity of the host, they soon attached to the branchiae, began to feed, and in the majority of cases remained there more or less permanently. Such males gradually became females.

THE FATE OF SUPERNUMERARY MALES AND FEMALES

The first series of experiments reported above furnish evidence that although any number of cryptonisci may attach to the same female and develop into juvenile males, only one male is allowed to reach maturity. This point has not been realized by most previous investigators except Caullery (1941) and Reverberi and Pitotti (1942), and hence, in the older literature; several cases of supernumerary males associated with one female bopyrid are mentioned, with the inference that they are highly unusual or evidence of polyandry. Pérez (1924), for example, reports three instances of this from his own observations involving *Pleurocrypta porcellanae* Hesse, *P. galathea* Hesse, and *Athelges lorifera* Hesse. In the light of recent work, these must be interpreted as cases of extra males in process of development before they have realized the full adult state, since all cases, when analyzed, resolve themselves into the stegophryxoid pattern, namely, one adult functional male accompanied by one or more smaller juvenile males or cryptonisci.

It must be concluded, therefore, that it is a general rule in the bopyrids that only one functional male is permitted at a time. Other potential males, temporarily tolerated, are expelled sooner or later. Whether the legitimate male, by virtue of its larger size, actually drives off the others, or whether they are repelled in some more refined manner is a question still to be answered.

Our observations on the question of excess females show that they, like the supernumerary males, are eliminated sooner or later, usually as juveniles. Only one adult female is ever found on an individual host.

One hermit crab, collected August 23, 1946, carried a large ovigerous Stegophryxus and had in addition four juvenile females in various stages of development attached to the abdomen and pleopods. This crab was isolated for daily observations and in less than a week's time the four juvenile females had disappeared. Two other cases of parasitized crabs, each with a juvenile female present in addition to the adult female Stegophryxus, were observed under laboratory conditions. One juvenile persisted from July 24 to August 10 and grew considerably in size before it was eliminated; the other lasted from July 29 to August 11.

It is significant that the lost juvenile females could not be found in the dish in which the crab had been isolated. Perhaps they drop off and are eaten by the crab. It is more probable, however, that they are driven off by the mature male. One finds, on occasion, the mature male wandering about on the outside of the female brood pouch, and it is not unlikely that the male engages in occasional forays over the abdomen of the crab and drives off or destroys the excess females before they reach maturity.

HISTOLOGICAL OBSERVATIONS ⁴

Four cryptonisci and ten males, the latter selected to form a graded series of sizes ranging from 1.2 mm. to 2.7 mm., were sectioned and studied histologically to determine the sexual nature of the larvae and the organogenesis of the male gonads.

⁴ The author gratefully acknowledges the assistance of Cornelius Sharbaugh, T.O.R., who, under our direction, prepared the slides and made the morphological studies on which this portion of the paper is based.

It was found that males of 2 mm.-length and over could be termed "adult" as judged by the length and development of the testes and the presence of spermatozoa in the vasa deferentia. In such males the reproductive organs are seen as a pair of long tubular masses, beginning in the first thoracic segment, and extending back into the seventh thoracic segment. The testes lie dorso-laterally adjoining the two liver tubes, one on each side of the animal, except for the anterior extremity of each, which occupies a ventro-lateral position in relation to the liver. In the sixth and seventh thoracic segments, the testes become vasa deferentia which open separately to the outside on the ventral surface of the seventh thoracic segment. The beginning of the vas deferens is often dilated to act as a temporary seminal vesicle.

The cells that make up the testis in the anterior-most portion of the organ are all of one type and equally distributed throughout the cross section. Elsewhere, spermatocytes, spermatids, and spermatozoa may be seen arranged in three distinct zones: spermatocytes in the inner zone next to the liver, spermatids in the middle, and spermatozoa in the outer zone (Fig. 4). The cells of the inner and middle zones are grouped into areas or patches, but those of the outer zone extend without interruption the entire length of the testis.

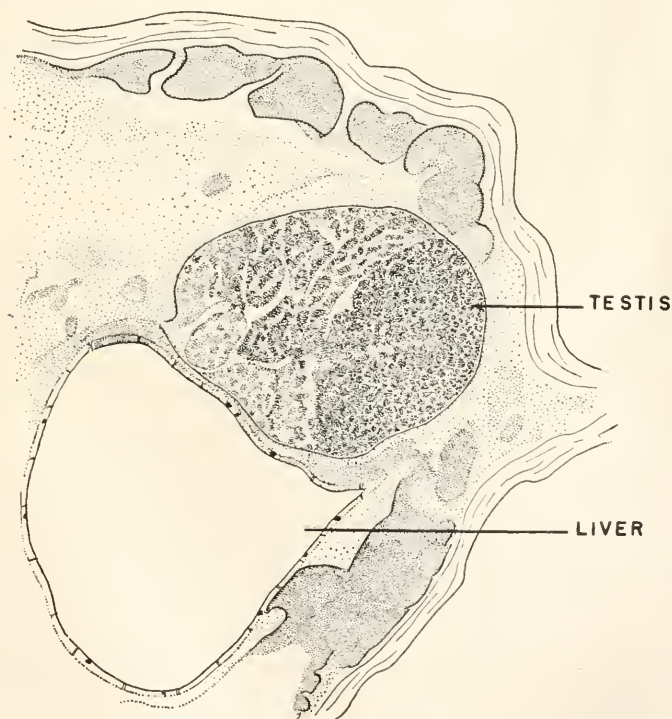


FIGURE 4. Right half of transverse section through the third thoracic segment of a male *Stegophryxus hyptius* measuring 2.7 mm. in length. The testis, dorso-lateral to the liver, shows the characteristic zonal arrangement: spermatocytes in the inner zone closest to the liver, spermatids intermediate in position, and spermatozoa in the outer zone. $\times 400$.

Males of approximately 1.5 mm.-length are essentially similar to the larger males except that the testes are shorter, beginning in the second or third thoracic segment, and the three characteristic zones appear only in the posterior portion. Anteriorly, the testes contain spermatocytes and spermatids, but no spermatozoa.

In the smallest male examined, length 1.2 mm., the testis on the left side was undeveloped; that on the right was short and contained spermatocytes and spermatids only. These occupied a relatively short middle section, with anterior and posterior extremities appearing empty.

None of the four cryptonisci examined, three in cross section and one in longitudinal section, showed either gonads or groups of cells that might be regarded as traces of gonads.

DISCUSSION AND CONCLUSIONS

The experiments reported on here seem to indicate that the cryptoniscus larvae of *Stegophryxus* can develop in either of two directions: into males under conditions of parasitism on a female bopyrid, or into females, under conditions of parasitism on a hermit crab. As in the case of *Bonellia* (Baltzer, 1914), the larvae are apparently indifferent, with both sex potencies. Which potency will be realized depends on the conditions of the environment.

It is evident that the female bopyrid exercises a masculinizing influence on the cryptonisci directly attached to it. This influence does not extend to cryptonisci which are merely in the neighborhood of the female. They receive no male stimulus. To be affected, the cryptoniscus must be in actual contact with the female and perhaps even imbibe her body fluids. Certainly they and the juvenile males receive nourishment from the female, or how else could they grow to increase as much as threefold in size?

Whether nutritive conditions alone provide the masculinizing stimulus, or whether the controlling influence is a specific substance of hormonal nature, is a question requiring further experimental study. Nourishment, as pointed out by Zimmer (1927), is probably the determining factor in the production of females, but for the production of males it seems necessary to assume, as in *Bonellia*, the transfer of an actual secretion from the body of the female to the larvae that are attached to her, which acts as a specific masculinizing substance.

The sex-determination theory proposed by Giard and Bonnier (1887), namely, that the first larva that invades a particular host transforms into a large female, while the next to come settles on this female and metamorphoses into a dwarf male, is an explanation entirely too simple; but the first assumption, at least, is supported by the results of our experiments. The fate of subsequent comers is less certain. Conceivably, a second cryptoniscus might arrive shortly after the first and also settle down to become an incipient female. There must obviously be a time interval of some days before the first-comer has metamorphosed sufficiently to invite the attention of new arrivals. Let us say, therefore, that the fate of subsequent comers is in no way different from the fate of any cryptoniscus; viz. those that settle directly on a crab become female-determined, while those that settle on a female of their species become male-determined.

One of the objections to the theory of Giard and Bonnier has been the fact that two females are sometimes found on the same host. Thus Hiraiwa (1936) says:

"If the female in the (branchial) cavity makes the later invader into male, why are two females found in one and the same cavity?"

The answer to this objection is now clear. A female does not influence the sex of later invaders unless they settle directly on her body. Should a cryptoniscus settle on the crab, no matter how close in position to a large female, the cryptoniscus will not be affected by this proximity so far as its sex-determination is concerned.

The sex determination theory of Smith (1909) and Goldschmidt (1920) with reference to the Bopyridae requires no discussion. It is eliminated by the facts presented in describing the life cycle of *Stegophryxus* and has previously been sufficiently criticized by Hiraiwa (1936).

Hiraiwa's own theory, disclaiming as it does differentiation due to environmental factors, is not in harmony with the results of the experiments reported here. His assumption, however, that the free-swimming larvae are not males but are sexually undifferentiated is in agreement with our findings.

Caullery's theory (1941) finds ample confirmation in the results of our experiments. Although the exact experimental verification he hoped for has not yet been realized by us, the converse experiment of transferring cryptonisci from the host to the female bopyrid has yielded satisfactory evidence that the sexes are not fixed from the start.

Coming now to the studies on *Ione thoracica* made by Reverberi and Pitotti (1942) and Reverberi (1947), and their relation to the observations and deductions reported here on *Stegophryxus hyptius*, we find when we tabulate the two for comparison (Table 1) that the same general pattern runs through both. Some, perhaps most, of the differences that do exist are modifications to be expected when comparing species of different genera that differ also in habits and habitat. Thus, since *Ione* is a branchial parasite, the larvae have the opportunity of settling on the gills of the host (to become female-determined), on the female parasite itself (to become male-determined), or on the skin of the host (likewise to become male-determined). *Stegophryxus* presents a simpler condition since the female is attached not to the branchiostegite but to the abdomen of the host. Therefore only two substrates are selected for attachment by the larvae: the abdomen of the host or the female herself. If abundant nourishment is the factor that determines females and less abundant nourishment, as Reverberi and Pitotti at first thought, determines males, it is hard to understand why the abdomen of *Pagurus* should furnish abundant nourishment to the cryptonisci of *Stegophryxus* while the abdomen of *Callianassa* should not likewise yield abundant nourishment to cryptonisci of *Ione*. Later (1947), Reverberi, as a result of further experiments, came to the conclusion that the larvae that attach to the female parasite are masculinized by a sex-determining substance produced by the female rather than by "less abundant nourishment"; but the problem of the so-called "complementary males" on the skin of *Callianassa* is still very puzzling.

Reverberi's experiments on *Ione* were made almost exclusively with the older post-cryptoniscid larvae; ours dealt almost entirely with earlier larvae in the cryptoniscus stage. Since the larvae on the body of *Callianassa* were already presumptive males, his chief experiments were to make females out of them. In *Stegophryxus*, on the other hand, the cryptonisci on the body of the host are presumptive females, hence our main experiments were to make males out of them. All in all, the two studies complement and illuminate each other. Together they

TABLE 1

A comparison between Ione thoracica and Stegophryxus hyptius with respect to sex-determination. Data for Ione compiled from the papers of Reverberi and Pitotti (1942) and Reverberi (1947)

<i>Ione thoracica</i>	<i>Stegophryxus hyptius</i>
1. Adult female lives in the branchial cavity of Callianassa.	1. Adult female lives on the abdomen of Pagurus.
2. Females result from cryptonisci that settle on the gill of the host.	2. Females result from cryptonisci that settle on the abdomen of the host.
3. Females secrete a diffusible substance capable of attracting free cryptoniscus larvae.	3. Same.
4. Cryptonisci that attach to the female become males.	4. Same.
5. Only one adult male is retained.	5. Same.
6. Cryptonisci that attach to the skin of the host become complementary males, capable of replacing the lost male of an adult pair.	6. Cryptonisci that attach to the skin of the host become juvenile females. Replacement of lost males occurs from free cryptonisci only.
7. Juvenile males, removed from adult females, can be transformed into females by placing them on the gills of the host.	7. No evidence that juvenile males can undergo sex reversal, but presumptive male cryptonisci could probably be transformed into females if they left the female and obtained nourishment from the host instead.
8. A widowed male can become female if it succeeds in attaching to the host gill and getting abundant nourishment.	8. It is unlikely that males, once differentiated, could undergo sex reversal.
9. The few comparable experiments reported did not yield results.	9. Presumptive female cryptonisci become males when removed from the host and placed on a female parasite.
10. Evidence supplied in 1947 that differentiated females can undergo sex reversal.	10. No evidence that differentiated females have the capacity for reversal to male.
11. Larvae that engorge host blood directly become females if nourishment is abundant (taken from gills), but become complementary males if nourishment is less abundant (taken from skin of host).	11. All larvae in a position to feed on the host directly become females. Only larvae that attach to the female become males.
12. Reverberi first attributed maleness, when larvae are attached to a female, to "less abundant food," but later (1947) explained it as due to masculinizing substance.	12. Data favors hypothesis of a masculinizing substance produced by the female.
13. Gonad tissue first appears in older post-cryptoniscid forms and the earliest gonad is indifferent.	13. Cryptonisci lack gonads. The earliest gonad in juvenile males is a testis. No data presented on the earliest type of gonad in a juvenile female.
14. Sex-determination is environmental, comparable (with modifications) to that described for Ophryotrocha.	14. Sex-determination is environmental, comparable (with modifications) to that described for Bonellia.

fulfill the expectations of Caullery and reveal a fundamental plan of sex-determination and sex-differentiation, a plan, however, that can be expected to exhibit minor variations when utilized by different genera of Bopyridae.

SUMMARY

Stegophryxus hyptius Thompson, an ectoparasite on the abdomen of the hermit crab *Pagurus longicarpus* Say, seeks the definitive host as a cryptoniscus larva.

The free-swimming cryptonisci are sexually undifferentiated and sexually undetermined. Differentiation follows fixation and is dependent on environmental factors. These conclusions are justified on the basis of the following observations and experiments.

1. Cryptonisci that settle directly on the host develop into females; those that attach to a female bopyrid develop into males.

2. Changes in the color pattern of the cryptonisci following fixation furnish criteria for judging the length of time they have been subjected to a particular environment.

3. Histological examination of the cryptoniscus shows that the gonads are not yet present as recognizable structures.

4. Presumptive female cryptonisci, if removed from the host at an early age and transferred to the brood pouch of a female *Stegophryxus*, will transform into males.

5. The failure of the converse experiment involving transfer of presumptive male cryptonisci from the female parasite to the host can definitely be laid to experimental difficulties.

6. The factor that determines maleness is a masculinizing substance imbibed with food from the female, but this substance does not act at a distance.

7. Attachment of supernumerary females as well as excess differentiating males is terminated sooner or later so that a particular crab is host to only a single adult female paired with one functional male.

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STUDIES ON MARINE BRYOZOA, III. WOODS HOLE REGION BRYOZOA ASSOCIATED WITH ALGAE

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INTRODUCTION

Heretofore there has been no extensive study made of the association between bryozoa and algae, except for the studies by Joliet (1877) and Prenant and Teissier (1924, 1927, 1932), although incidental association notes are scattered throughout taxonomic papers.

The purposes of the present study are several: (1) to note any association that may exist between algae and certain bryozoa (bryozoa likely to be encountered in the Woods Hole region); (2) to aid collectors of bryozoa, since in some instances by collecting specified algae one is almost sure to find a number of desired bryozoa; (3) to make any observations possible on the tentacle number, the occurrence of embryos, larvae and ovicells in specimens collected during the summer months, and (4) to report any additional species from the collection area.

COLLECTION DATA

The materials used in this study were marine algae of three general groups: (1) freshly collected specimens; (2) dry, pressed specimens mounted at various times in the past on herbarium sheets; and (3) specimens preserved in the Botany Course Stock Collection of the Marine Biological Laboratory (M. B. L.) of Woods Hole, Mass. More species were examined than are here recorded but only those 37 algal species which had bryozoa growing on them are here listed.

Some of the fresh material was obtained in the intertidal zone by shore collecting; some had drifted in from deeper waters some distance from shore; and some had been dredged with a scallop dredge from waters about 20 to 60 feet in depth.

Some of the material was collected by the authors, some by the M. B. L. Invertebrate Zoology and Botany classes on their field trips, some by the M. B. L. Supply Department and Collecting Crew, and one algal species by Dr. Maxwell Doty of Northwestern University. To all these the authors wish to express their most sincere appreciation, and especially to Dr. William Randolph Taylor of the University of Michigan for very helpful suggestions, continued kindly interest, and for specimens of *Membranipora tuberculata* and their algal hosts from his own collection.

The *Laminaria longicuris* was brought in by Dr. Doty from Race Point, near Provincetown, Mass. on VIII-18-1947. The Rye Ledge, Rye, New Hampshire specimens of *Chondrus crispus*, *Phycodrys rubens* and *Phyllophora membranifolia* were collected by the junior author on IX-30-1945. The New Rochelle, New York specimens of *Ascophyllum nodosum*, *Ascophyllum Mackaii*, *Chondrus crispus* and *Laminaria Agardhii* were observed on IX-16 and 22-1945, and on X-3-1946 by the senior author. The remainder of the specimens were collected in the waters around and between North Falmouth, Martha's Vineyard, Woods Hole, Vineyard Sound, Buzzards Bay, Penikese Island and New Bedford, all in Massachusetts, during the summer months, between June 30 and August 31 approximately, over a period of several years (Sept. 1874, 1916, 1930, 1935, 1936, 1938, 1939, 1944 through 1947). Specimens from the five earliest years came from the M. B. L. Botanical Collection, some from wet mounts and some from dried herbarium mounts. Exact records, dates and collection numbers of all these algal specimens are on file, but only a very condensed amount of these data is included in Tables I to IV, to save space.

The senior author identified the bryozoa, the junior author classified the algae and collected many of them.

TABLE I
Collection sites and bryozoa associated with green algae

	<i>Cladophora gracilis f. tenuis</i>	<i>Enteromorpha intestinalis</i>	<i>Ulva Lactuca var. rigida</i>	Total No. green algae having this bryozoan species
<i>Bowerbankia gracilis</i>		x	x	2
<i>Bugula turrita</i>	x	x	x	3
<i>Cryptosula pallasiana</i>		x	x	2
<i>Flustrella hispida</i>			x	1
<i>Pedicellina cernua</i>	x			1
Total No. bryozoan spp. on this alga	2	3	4	
Collected at Woods Hole, Mass.	x	x	x	

To date there have been reported 84 species of marine bryozoa from the Woods Hole region, by Osburn (1912) mostly, and by Rogick (1945a, 1948). Some of the Woods Hole species have been previously reported from such widely separated regions as the coast of Africa, Australia, the Azores, Brazil, Denmark, Great Britain, Japan, New Zealand, Panama, Zanzibar, and the Pacific coast of North America. These were reported from various substrates as shells, rocks, hydroids, algae, various animals, piles, and other submerged objects. The present study was mainly directed toward finding the exact algal species on which bryozoa grow. Previous reports from the Woods Hole region occasionally did indicate the algal genera but rarely the species on which the bryozoa occurred. The following lists of algae and bryozoa are of species collected or examined for the present paper.

Below follows a list of 37 algae on which were commonly found various bryozoans. Taylor's (1937) classification is used.

TABLE II
Collection sites and bryozoa associated with brown algae

	<i>Ascopyllum Mackaii</i>	<i>Ascopyllum nodosum</i>	<i>Chorda Filum</i>	<i>Cladostephus verticillatus</i>	<i>Desmarestia aculeata</i>	<i>Fucus evanescens</i>	<i>Fucus vesiculosus</i>	<i>F. vesiculosus spiralis</i>	<i>Laminaria Agardhii</i>	<i>Laminaria longicirris</i>	<i>Sargassum Filipendula</i>	Total No. brown algal spp. associated with this bryozoan
<i>Aetea sica</i>		x	x	x			x	x	x		x	7
<i>Aeverrillia armata</i>									x			1
<i>Aeverrillia setigera</i>		x							x		x	3
<i>Alcyonidium polyoum</i>									x	x		2
<i>Bowerbankia gracilis</i>		x	x	x			x	x			x	6
<i>Bowerbankia imbricata</i>							x		x		x	3
<i>Bugula cucullifera</i>						x				x		2
<i>Bugula flabellata</i>							x					1
<i>Bugula turrita</i>		x	x				x	x			x	6
<i>Callopora aurita</i>									x			1
<i>Cribrilina punctata</i>									x			1
<i>Crisia eburnea</i>		x			x				x		x	4
<i>Cryptosula pallasiana</i>	x	x						x	x			4
<i>Electra hastingsae</i>								x	x			2
<i>Electra pilosa</i>		x			x	x		x	x	x	x	7
<i>Flustrella hispida</i>		x					x					2
<i>Hippothoa hyalina</i>		x						x	x		x	4
<i>Membranipora lacroixii(?)</i>	x											1
<i>Microporella ciliata</i>									x			1
<i>Pedicellina cernua</i>		x					x		x			3
<i>Schizoporella biaperta</i>					x				x			2
<i>Schizoporella unicornis</i>								x	x			2
<i>Scruparia ambigua</i>					x	x			x			3
<i>Scruparia clavata</i>									x			1
<i>Smittina trispinosa</i>									x			1
Total No. bryozoan spp. found on this alga	2	10	3	2	4	3	7	8	20	3	8	
Collected at Woods Hole		x	x	x			x	x	x		x	
Collected at North Falmouth		x						x			x	
Collected in Vineyard Sound			x		x	x					x	
Collected at Martha's Vineyard					x	x	x		x			
Collected at Penikese Island								x				
Collected at Provincetown										x		
Collected at New Rochelle	x	x							x			

TABLE III
Collection sites and bryozoa associated with red algae

	<i>Agardhiella lenera</i>	<i>Calothrix roseum</i>	<i>Ceramium rubiforme</i>	<i>Ceramium rubrum</i>	<i>Champia parvula</i>	<i>Chondrus crispus</i>	<i>Corallina officinalis</i>	<i>Cyphopleura</i> sp.	<i>Cystoclonium purpureum</i> <i>chryosum</i>	<i>Gracilaria confervoides</i>	<i>Gracilaria foliifera</i>	<i>Lithothamnium</i> sp.	<i>Phycodrys rubens</i>	<i>Phyllophora Brodiaei</i>	<i>Phyllophora membranifolia</i>	<i>Planaria sericea</i>	<i>Polysides rotundus</i>	<i>Polysiphonia elongata</i>	<i>Polysiphonia nigra</i>	<i>Polysiphonia nigrescens</i>	<i>Polysiphonia variegata</i>	<i>Rhodomela subfusca</i>	<i>Rhodomenia palmata</i>	Total No. red algal spp. associated with this bryozoan
<i>Aetea sica</i>				X		X			X				X	X	X					X				11
<i>Aenerrillia armata</i>																								1
<i>Aenerrillia setigera</i>																								1
<i>Alcyonidium polyomm</i>					X	X							X	X	X	X								5
<i>Bowerbankia gracilis</i>						X					X													3
<i>Bowerbankia imbricata</i>						X					X													10
<i>Bugula cucullifera</i>	X																							2
<i>Bugula turrita</i>						X							X	X	X	X								2
<i>Callopora aurita</i>																								2
<i>Cellepora dichotoma</i>										X														7
<i>Cribrilina annulata</i>																								2
<i>Cribrilina punctata</i>																								4
<i>Crista eburnea</i>						X							X	X	X	X								6
<i>Cryptosula pallasiana</i>				X		X			X				X	X	X	X								14
<i>Electra pilosa</i>						X			X				X	X	X	X								5
<i>Flustrella hispida</i>						X			X				X	X	X	X								10
<i>Hippoporina contracta</i>						X			X				X	X	X	X								2
<i>Hippothoa hyalina</i>				X		X			X				X	X	X	X								2
<i>Lichenopora hispida</i>						X			X				X	X	X	X								13
<i>Membranipora lacroixii(?)</i>						X			X				X	X	X	X								2

TABLE III—Continued

	<i>Agardhiella tenera</i>	<i>Callithamnion roseum</i>	<i>Ceramium rubrifforme</i>	<i>Ceramium rubrum</i>	<i>Champia parvula</i>	<i>Chondrus crispus</i>	<i>Corallina officinalis</i>	<i>Cyphopleura</i> sp.	<i>Cystoclonium purpureum</i> <i>citrhosum</i>	<i>Gracilaria confervoides</i>	<i>Gracilaria foliifera</i>	<i>Lithothamnium</i> sp.	<i>Phycodrys rubens</i>	<i>Phyllophora Brodiaei</i>	<i>Phyllophora membranifolia</i>	<i>Plumaria sericea</i>	<i>Polysides rotundus</i>	<i>Polysiphonia elongata</i>	<i>Polysiphonia nigra</i>	<i>Polysiphonia nigrescens</i>	<i>Polysiphonia variegata</i>	<i>Rhodomela subfusca</i>	<i>Rhodomenia palmata</i>	Total No. red algal spp. associated with this bryozoan
<i>Membranipora tuberculata</i> <i>Microporella ciliata</i> <i>Pedicellina cernua</i> <i>Schizoporella biaperta</i> <i>Schizoporella unicomis</i> <i>Scruparia ambigua</i> <i>Smittina trispinosa</i>						X		X			X			X	X	X						X	1	
						X			X				X	X	X	X				X			4	
						X	X		X											X			7	
						X	X		X				X							X			4	
						X	X		X														8	
						X	X		X														7	
						X	X		X														3	
Total No. bryozoan spp. on this alga	1	1	1	5	3	20	4	1	11	4	3	1	10	15	23	3	2	1	3	7	8	4	7	
Collected at Woods Hole Collected at Martha's Vineyard Collected in Vineyard Sound Collected at North Falmouth Collected on Penikese Island Collected at New Bedford Collected at New Rochelle Collected at Rye, N. H. From Puerto de la Paloma, Uruguay*	X	X	X	X	X	X	X			X	X	X		X	X	X		X		X	X	X		
									X															

* Specimens sent by Dr. W. R. Taylor.

TABLE IV
Collection sites and other Bryozoan data

	Penikese Island, Mass.	New Bedford, Mass.	North Palmouth, Mass.	Vineyard Sound, Mass.	Martha's Vineyard, Mass.	Woods Hole, Mass.	New Rochelle, New York	Rye, New Hampshire	Provincetown, Mass.	Puerto de la Paloma, Uruguay	No. of algal spp. from which bryozoan was reported in Tables I, II, III	Partial list of the No. of algal spp. or genera from which this bryozoan was reported by other workers. See under each bryozoan species discussion	Tentacle number. Personal observations started, others started	In filled-ovicell, embryo-producing or larva-releasing stage at Woods Hole
1. <i>Aetea sica</i>			X	X	X	X					18		9-11	July 30-Aug. 6±
2. <i>Aeerrillia armata</i>		X			X						2		8	
3. <i>Aeerrillia setigera</i>		X				X	X		X		8		8	
4. <i>Alecyonidium polyoum</i>		X				X					5	5	16, 20*	
5. <i>Bowerbankia gracilis</i>		X	X		X	X					18	6	8	
6. <i>Bowerbankia imbricata</i>		X		X	X	X	X		X		5		10	All of Aug. ±
7. <i>Bugula cucullifera</i>					X						4		13	Aug. 1-18 ±
8. <i>Bugula flabellata</i>			X	X	X	X					1			June 1-Nov. 15
9. <i>Bugula turrita</i>					X	X		X			16		14	June 30-Aug. 15±
10. <i>Callopora aurita</i>					X	X					3			July 25±
11. <i>Cellepore dichotoma</i>					X	X		X			4			
12. <i>Cribrilina annulata</i>					X	X		X			1	1		
13. <i>Cribrilina punctata</i>					X	X		X			7			
14. <i>Crisia eburnea</i>	X	X	X	X	X	X		X			18	2	8*	Aug. 8±
15. <i>Cryptosula pallasiana</i>			X		X	X	X				11	4	16	June-Sept. ±

TABLE IV—Continued

	Penikese Island, Mass.	New Bedford, Mass.	North Falmouth, Mass.	Vineyard Sound, Mass.	Martha's Vineyard, Mass.	Woods Hole, Mass.	New Rochelle, New York	Rye, New Hampshire	Provincetown, Mass.	Puerto de la Paloma, Uruguay	No. of algal spp. from which bryozoan was reported in Tables I, II, III	Partial list of the No. of algal spp. or genera from which this bryozoan was reported by other workers. See under each bryozoan species discussion	Tentacle number. Personal observations unless starred, others starred	In filled-ovicell, embryo-releasing stage at Woods Hole
16. <i>Electra hastingsae</i>						X	X	X	X		2	1	12-14	
17. <i>Electra pilosa</i>	X	X	X	X	X	X	X	X	X		17	8	13	
18. <i>Flustrella hispida</i>						X					5	3	12*	
19. <i>Hippoporina contracta</i>						X		X			2		12*	
20. <i>Hippothoa hyalina</i>			X	X	X	X					17	5	12*-14*	July-Aug. ±
21. <i>Lichenopora hispida</i>	X	X						X			2			
22. <i>Membranipora lacroixii</i> (?)							X				3		11	
23. <i>Membranipora tuberculata</i>										X	1	3		
24. <i>Microporella ciliata</i>					X	X					5	3	13*-14*	Aug. ±
25. <i>Pedicellina cernua</i>		X	X	X	X	X	X				11	6	8-12; 8*-24*	
26. <i>Schizoporella biaperta</i>		X	X	X	X	X					9		12	July-Aug. ±
27. <i>Schizoporella unicornis</i>		X	X	X	X	X					6	3	about 18*	July-Aug. ±
28. <i>Scruparia ambigua</i>			X		X	X					11		10	
29. <i>Scruparia clavata</i>			X		X	X					1			
30. <i>Smittina trispinosa</i>			X		X	X					4			

* The references for the starred* numbers in the Tentacle No. column can be found under each species' descriptive section.

± The plus and minus signs in the last column mean that embryos or larvae very likely were present earlier or later than the days and months listed in the last column.

LIST OF COLLECTED ALGAE

CHLOROPHYCEAE (green algae)

1. *Cladophora gracilis* (Griffiths) Kützing, forma *tenuis* Farlow
2. *Entromorpha intestinalis* (Linnaeus) Link (a proliferous form)
3. *Ulva Lactuca* Linnaeus var. *rigida* (C. Agardh) LeJolis

PHAEOPHYCEAE (brown algae)

4. *Ascophyllum Mackaii* (Turner) Holmes et Batters
5. *Ascophyllum nodosum* (Linnaeus) LeJolis
6. *Chorda Filum* (Linnaeus) Lamouroux
7. *Cladostephus verticillatus* (Lightfoot) C. Agardh
8. *Desmarestia aculeata* (Linnaeus) Lamouroux
9. *Fucus evanescens* C. Agardh
10. *Fucus vesiculosus* Linnaeus
11. *Fucus vesiculosus* var. *spiralis* Farlow
12. *Laminaria Agardhii* Kjellmann
13. *Laminaria longicuris* De la Pylaie
14. *Sargassum Filipendula* C. Agardh

RHODOPHYCEAE (red algae)

15. *Agardhiella tenera* (J. Agardh) Schmitz
16. *Callithamnion roseum* (Roth) Harvey
17. *Ceramium rubriforme* Kylin
18. *Ceramium rubrum* (Hudson) C. Agardh
19. *Champia parvula* (C. Agardh) Harvey
20. *Chondrus crispus* (Linnaeus) Stackhouse
21. *Corallina officinalis* Linnaeus
22. *Cryptopleura* sp.
23. *Cystoclonium purpureum* (Hudson) Batters var. *cirrhosum* Harvey
24. *Gracilaria confervoides* (Linnaeus) Greville
25. *Gracilaria foliifera* (Forsskål) Børgesen
26. *Lithothamnium* sp.
27. *Phycodrys rubens* (Hudson) Batters
28. *Phyllophora Brodiaei* (Turner) J. Agardh
29. *Phyllophora membranifolia* (Goodenough et Woodward) J. Agardh
30. *Plumaria sericea* (Harvey) Ruprecht
31. *Polyides rotundus* (Gmelin) Greville
32. *Polysiphonia elongata* (Hudson) Harvey
33. *Polysiphonia nigra* (Hudson) Batters
34. *Polysiphonia nigrescens* (Hudson) Greville
35. *Polysiphonia variegata* (C. Agardh) Zanardini
36. *Rhodomela subfusca* (Woodward) C. Agardh
37. *Rhodymenia palmata* (Linnaeus) Greville

Below is a list of the 30 bryozoan species which were found growing on the various algae examined by the authors.

LIST OF COLLECTED BRYOZOA

ENTOPROCTA

1. *Pedicellina cernua* (Pallas) 1771

ECTOPROCTA

Cyclostomata or Stenolaemata

2. *Crisia eburnea* (Linnaeus) 1758
3. *Lichenopora hispida* (Fleming) 1828

Ctenostomata

4. *Acyroverrillia armata* (Verrill) 1873
5. *Acyroverrillia setigera* (Hincks) 1887
6. *Alcyonidium polyomm* (Hassall) 1841
7. *Bowerbankia gracilis* Leidy 1855
8. *Bowerbankia imbricata* (Adams) 1800
9. *Flustrella hispida* (Fabricius) 1780

Cheilostomata

10. *Actea sica* (Couch) 1844
11. *Bugula cucullifera* Osburn 1912
12. *Bugula flabellata* (Thompson) 1848
13. *Bugula turrita* (Desor) 1848
14. *Callopora aurita* (Hincks) 1877
15. *Cellepora dichotoma* Hincks 1862
16. *Cribrilina annulata* (Fabricius) 1780
17. *Cribrilina punctata* (Hassall) 1841
18. *Cryptosula pallasiana* (Moll) 1803
19. *Electra hastingsae* Marcus 1938
20. *Electra pilosa* (Linnaeus) 1767
21. *Hippoporina contracta* (Waters) 1899
22. *Hippothoa hyalina* (Linnaeus) 1767
23. *Membranipora lacroixii* (?)
24. *Membranipora tuberculata* (Bosc) 1802
25. *Microporella ciliata* (Pallas) 1766
26. *Schizoporella biaperta* (Michelin) 1842
27. *Schizoporella unicornis* (Johnston) 1847
28. *Scruparia ambigua* (d'Orbigny) 1841
29. *Scruparia clavata* Hincks 1857
30. *Smittina trispinosa* (Johnston) 1825

BRYOZOAN GROWTHS ON ALGAE

The bryozoa form white, grey, yellow, salmon-pink, or brown growths on the algae. Some bryozoan colonies are thin, flat, encrusting and closely adherent. Others are dendritic, arborescent, or may form a fuzzy mass of tiny vesicles. Still others coat the algae with a gelatinous, rubbery, or leathery film. The calcareous

bryozoa often retain their zooecial patterns and specific characteristics pretty well even though the algal host specimens have been dried and pressed in the normal course of herbarium sheet mounting. It was no harder to identify *Hippothoa hyalina* from a dry 1874 herbarium mount of *Phycodrys rubens* than from a freshly collected alga.

Bryozoa grow on various parts of the algal plant. The holdfast processes of Laminaria and related forms are excellent sites for attachment of at least 21 hard, horny, or soft bryozoan species. Bryozoa grow on and between the holdfast processes as well as on the rocks to which the holdfasts adhere. Laminaria and Rhodymenia blades are favorite attachment sites for *Electra pilosa* which is very common and especially abundant on these algae, sometimes coating both sides of the entire blade for an area of several inches with a thin, frosty-white, single-layered cover of contiguous bryozoan colonies. *Membranipora tuberculata* has the same habit of extensively encasing its algal hosts with the fine bryozoan mesh.

The basal or most proximal parts of *Chondrus crispus* and Phyllophora are encrusted by many bryozoans like Aeverillia, Bowerbankia, *Cellepora dichotoma* and *Hippothoa hyalina*, while the most distal tips are somewhat less often utilized for bryozoan attachment. Sometimes, if the bryozoan growth is especially rich or dense on these two algal genera, the whole blade may be covered. Alcyonidium may encase a whole blade and sometimes extend even beyond the tips of the plant. The two Schizoporellae also may grow so readily as to produce shelf-like extensions of the colony beyond the plant thallus.

The basal parts of Ascophyllum and Fucus are generally favored by the bryozoan colonies, as are the crevices and depressions around the airbladders and where branches originate. *Flustrella hispida* and Bowerbankia particularly favor these plants.

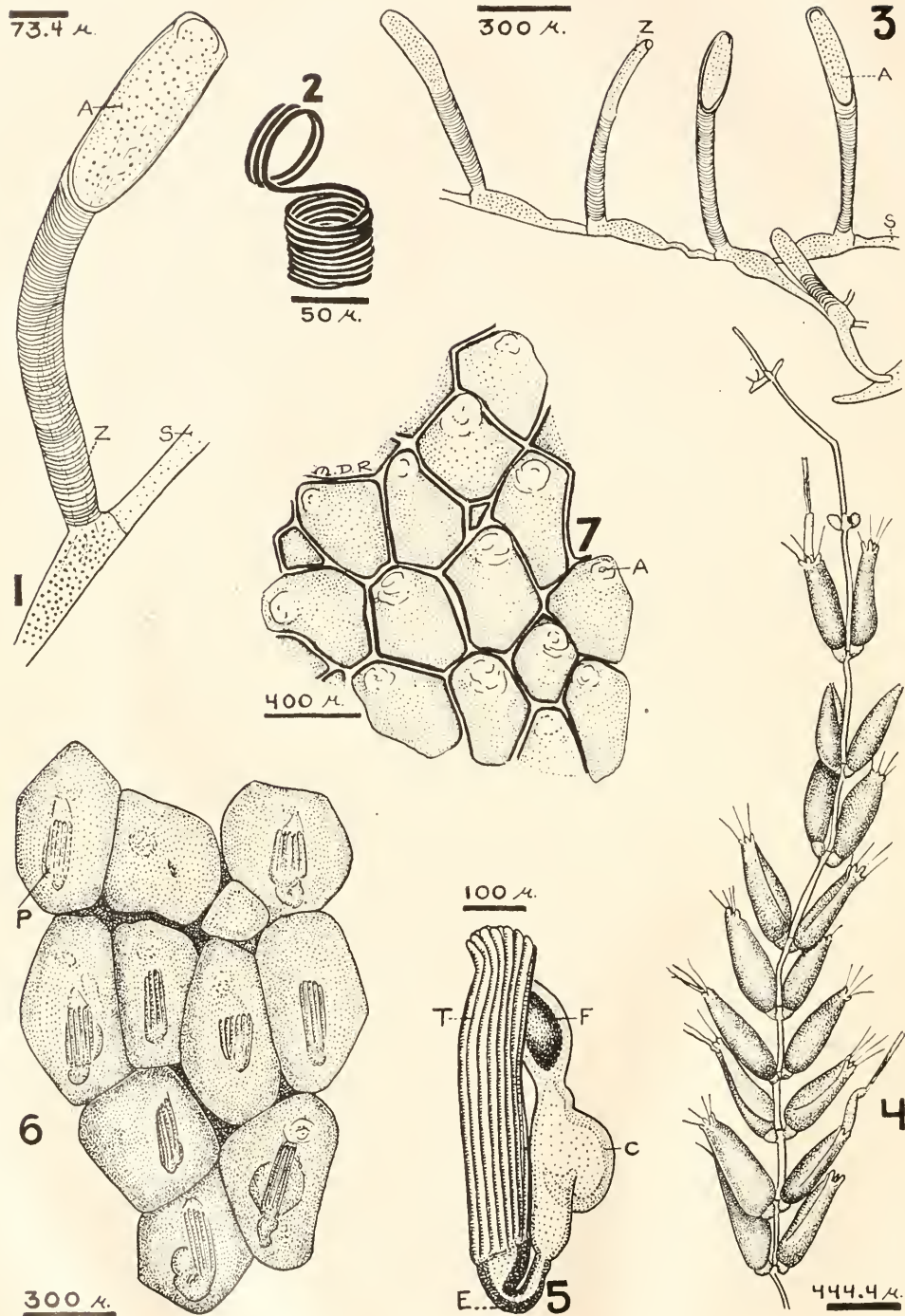
The few zoaria (bryozoan colonies) found on the green algae generally were small, consisting of only a few zooids, and did not produce such luxuriant and extensive growths as did the species which grew on the browns and reds.

The zoaria, as a rule, were one layer in thickness on the algae, with the exception of occasional specimens of *Schizoporella biaperta*, *S. unicornis* and *Smittina trispinosa*, which might be laminate. The laminate condition is more common on the firmer substrates (rocks) than on algae. *Hippothoa hyalina* and *Electra pilosa* were always single-layered on the plants.

Of the six most frequently encountered bryozoa (*Actea sica*, *Bowerbankia gracilis* and *Crisia churrua* each on 18 algal species, *Electra pilosa* and *Hippothoa hyalina* each on 17, and *Bugula turrita* on 16 algal species) the least conspicuous is *Actea*. It readily escapes detection unless the alga is examined microscopically. Because of their very characteristic growth habit and general appearance, *Electra*, *Bugula*, *Bowerbankia* and *Crisia* can be recognized with the unaided eye. *Hippothoa*, with a little practice, becomes recognizable because it forms small, short, calcareous, white sheaths around the thin algal stalks and filaments. *Hippothoa* especially accommodates itself readily to the smallest filaments and branches.

The bryozoa occurred in close association on the same algal thallus with many other animal forms. Numerous shells of *Spirorbis* sp. grew alongside the *Lichenopora hispida* from Rye, N. H. Sponges, hydroids, annelid worm tubes, *Botryllus schlosseri*, *Molgula manhattensis*, Styela, Foraminifera, and several species of bryozoa were sometimes found on a heavily populated alga. Hydroids,

PLATE I



Foraminifera, and several bryozoan species often were found on the same blade of *Ascophyllum*, *Chondrus*, *Laminaria*, *Phycodrys*, or *Phyllophora*. *Aeverrillia* and *Aetea* would sometimes grow on *Bugula* and hydroid colonies as well as on algal thalli.

The tentacle number and the time of larval production were obtained for some species but not for all because sometimes the colonies died before they could be examined, and sometimes the organisms were so exasperatingly slow in extending their tentacles for a count. Such data as could be obtained are listed in Table IV and also in the species' descriptive section which follows.

AETEA SICA

(Figures 1-3)

Aetea sica is fairly common, although not reported from this region previously. It forms a thin, white, bristly tracery on 18 different algal species. The zooids resemble fine upright tubes just big enough to be seen with the unaided eye. Slender stolons connect the bases of the upright zooids and adhere closely to the substratum (Fig. 3). Nine to eleven tentacles were counted in a few zooids. Ovicells were filled with live developing pinkish larvae from at least July 31 through August 6.

The feature by which Marcus (1937, p. 29) distinguishes *Aetea sica* from the previously reported *Aetea anguina* is the ratio of the aperture (opesium) length to opesium width. The opesial ratio for *A. anguina* is between 1.7:1 and 2:1. For *A. sica* it is between 2.6:1 and 4:1. In Figure 1, one zooid has a 4:1 ratio. If the ratio is a valid characteristic of the two species, then some of the previously reported *Aetea anguina* from the Woods Hole area must belong to *Aetea sica*.

PLATE I *

FIGURE 1. *Aetea sica*. Upright zooid (Z) growing from a punctate stolon (S) enlargement. The opesium (A) of this zooid is about three times as long as wide (a 3:1 ratio). The scale above applies to this figure. Hadley Harbor specimens, VII-28-1939.

FIGURE 2. *Aetea sica*. Detail of a broken stalk.

FIGURE 3. *Aetea sica*. A colony of five zooids (Z) arising from stolons (S). The upper right zooid has an opesium (A) about four times longer than wide (4:1 opesial ratio). The scale above applies to this figure.

FIGURE 4. *Aeverrillia armata*. A sprig of a colony collected from Lagoon Pond, Martha's Vineyard, VIII-17-1945.

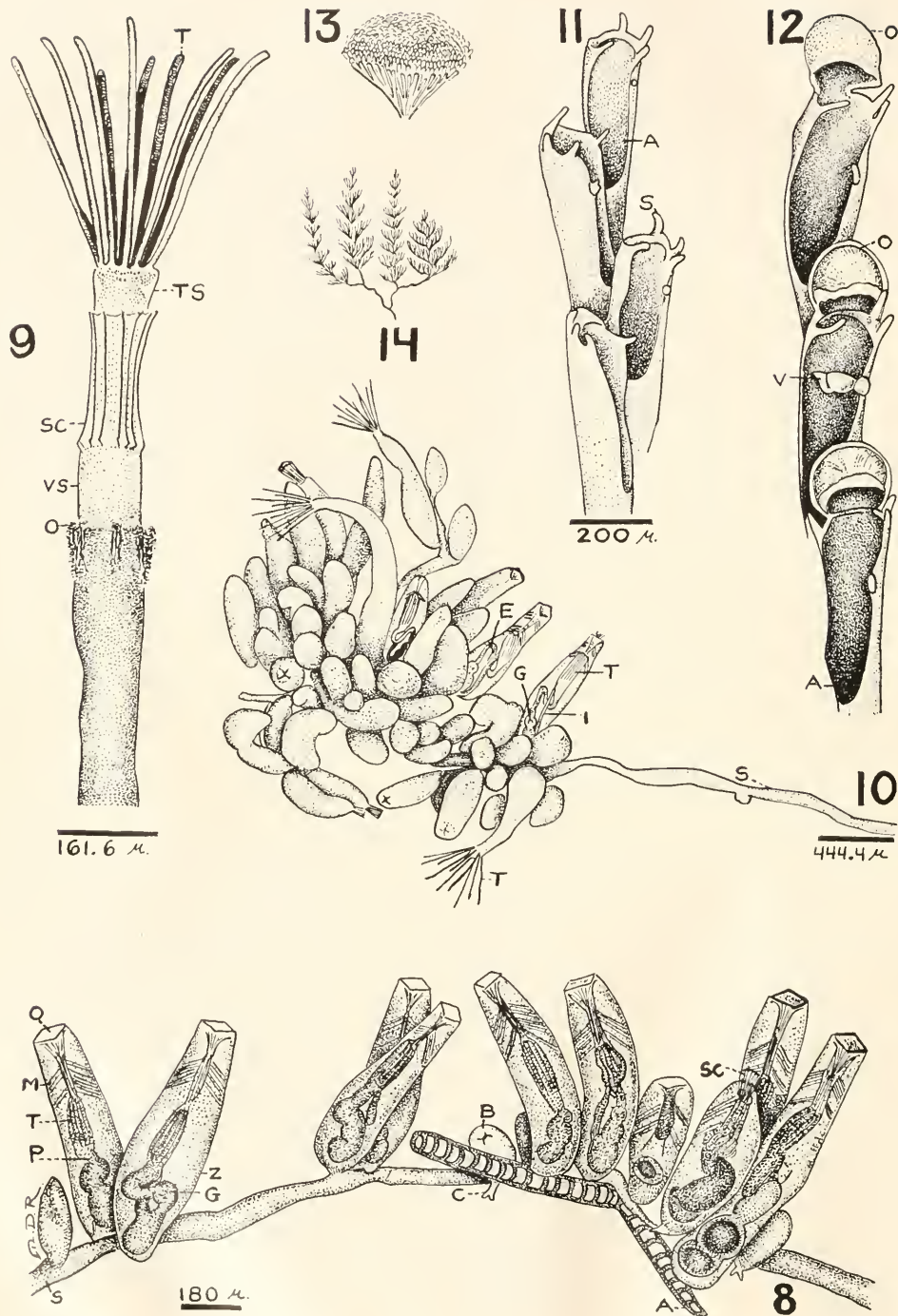
FIGURE 5. *Alcyonidium polyomm*. A polypide torn out of the colony, in its natural withdrawn position. It consists of tentacles (T), esophagus (E), caecum (C) and rectum which in this sketch contains a large dark fecal pellet (F). Collected off Davenport Park, New Rochelle, N. Y., on IX-22-1945.

FIGURE 6. *Alcyonidium polyomm*. Part of a young, fairly transparent colony most of whose zoecia contain a sketchily outlined withdrawn polypide (P). Of the same date and collecting locality as specimens of the preceding figure.

FIGURE 7. *Alcyonidium polyomm*. Somewhat thicker-walled colony, with slightly raised orifices (A). Collected at Black Rock, New Bedford Harbor, on VIII-8-1945.

* Figures on all plates, with the exception of Figures 13, 14 and 28, were drawn with the aid of a camera lucida. The species are alphabetically arranged except for Figure 14.

PLATE II



AEVERRILLIA ARMATA

(Figure 4)

Aeverrillia armata is transparent, yellowish, and horny, and occurs on *Laminaria Agardhii* and *Phyllophora membranifolia*. The latter alga was heavily encrusted with ten other bryozoan species and several algal species. *Aeverrillia armata* consists of numerous slender, paired autozooids arising from narrow stolons which cling closely to the plant but which can be pulled off as slender threads. This species is very similar to *A. setigera* which was discussed very fully in a previous study (Rogick, 1945a), except that it lacks the basal clasping processes of *A. setigera*. The polypides have eight tentacles in both species of the genus.

AEVERRILLIA SETIGERA

This delicate bryozoan was found growing inconspicuously on eight algal species. It clings closely to the plant thallus. It was pictured adequately in the previous study (Rogick, 1945a), so no figure of it is here included. The resemblance between it and *A. armata* is so close that one could easily mistake the one for the other.

ALCYONIDIUM POLYOUM

(Figures 5-7)

The various Alcyonidia are difficult to tell apart. The present *Alcyonidium polyoum* forms a firm gray or sometimes slightly yellowish crust around the hold-

PLATE II'

FIGURE 8. *Bowerbankia gracilis*. An uncrowded stolonate colony of nine full-grown zooids (Z) and five smaller buds (B), growing on an algal filament (A). Other structures shown are: (C) caudal process; (G) gizzard; (M) parieto-vaginal musculature; (O) squared orifice; (P) polypide; (S) stolon; (SC) setigerous collar. Collection site and date same as for Figure 7.

FIGURE 9. *Bowerbankia imbricata*. Upper part of an extruded polypide showing ten tentacles (T) which upon retraction can be withdrawn into the tentacular sheath (TS). Around that is a stiff transparent setigerous collar (SC) which in turn can be withdrawn into the vestibular sheath (VS). Some debris has accumulated on the edge of the squared orifice (O). From Glen Island, New Rochelle, N. Y. on IX-16-1945.

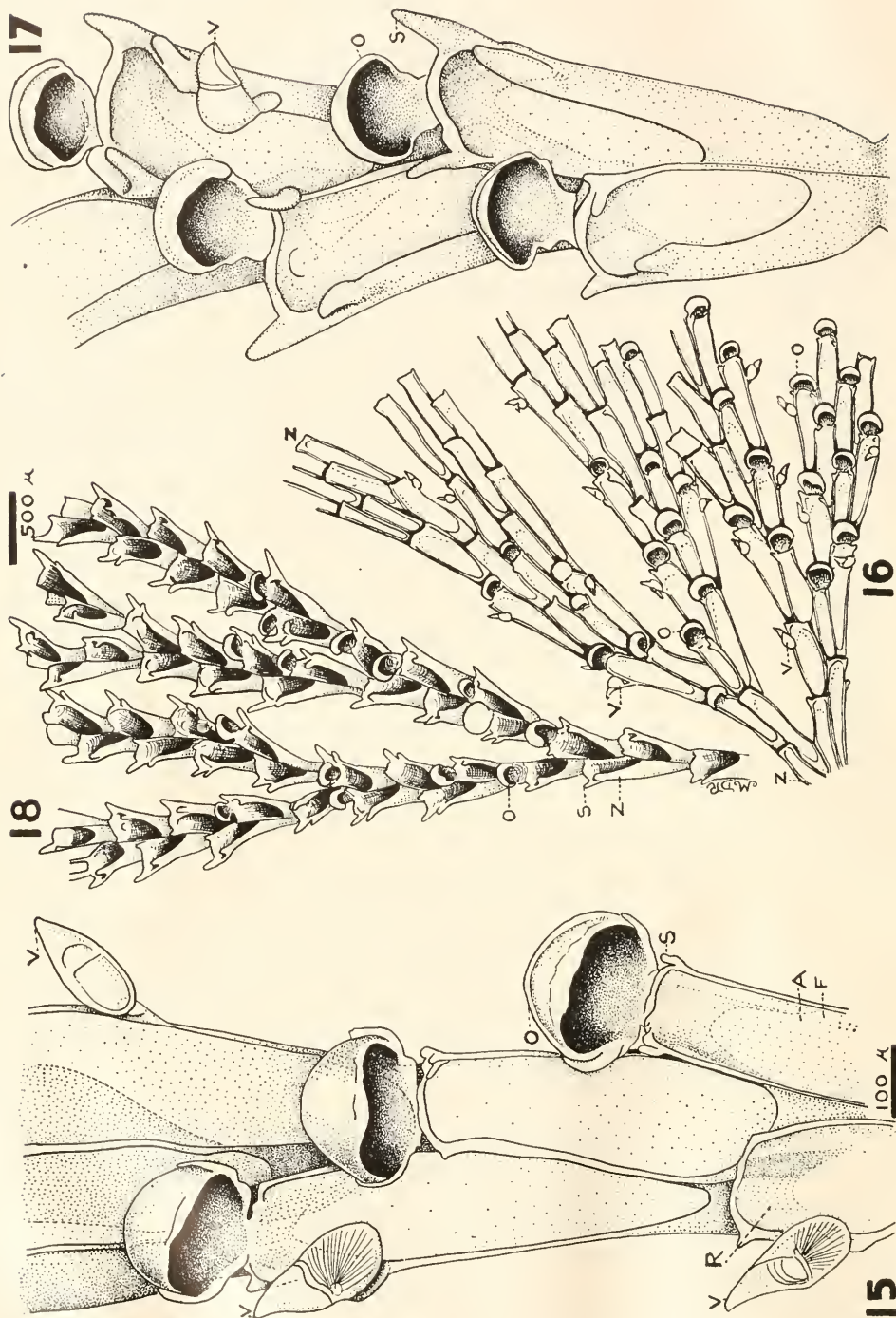
FIGURE 10. *Bowerbankia imbricata*. A crowded colony which was scraped from *Chondrus crispus*. Collection date and site the same as for the preceding figure. Three of the long zooids have their tentacles extended. Three smaller ones have their setigerous collars partly extruded. Three zooids are shown with the polypides within them. The following parts are labelled: (E) esophagus; (G) gizzard; (I) intestine; (S) stolon; (T) tentacles.

FIGURE 11. *Bugula cucullifera*. Four zooecia, each provided with four spines (S). The upper three zooids show, at the side of the opesium (A), the remains of the short peduncle which had borne an avicularium. From Provincetown, Mass., on VIII-18-1947; Dr. M. Doty collector.

FIGURE 12. *Bugula cucullifera*. Three fertile zooecia topped with ovicells (O). The middle zooecium bears an avicularium (V), the other two have lost theirs. (A) is the opesium. The second row of zooecia which normally would be at the side of these zooecia was incomplete and was therefore not shown here. Same collection date and site and drawn to the same scale as the preceding figure.

FIGURE 13. *Bugula flabellata*. A freehand sketch, showing the close tuft-like mode of colony growth. About natural size.

FIGURE 14. *Bugula turrita*. A freehand sketch, showing the dainty, open spiral mode of colony growth. About natural size.



fasts, stalks, and blades of at least five algal species. Prenant and Teissier (1924, pp. 23, 27) reported *Alcyonidium* from *Ascophyllum*, *Chondrus*, *Fucus*, *Himantalia*, *Laminaria* (*saccharina*?) and *Saccorhiza bulbosa*. Sometimes it coats the entire alga, using the various branches as cores around which to grow. The colony is rubbery to the touch.

The polypides had 16 tentacles. Measurements for 18 zooids ranged thus: zooid length 0.36–0.648 mm. and zooid width 0.24–0.504 mm. These are similar in range to figures given by Harmer (1915, pp. 37–38). The extremes in tentacle number given by various authors are 12 (Harmer, 1915, p. 38) to 20 (Silén, 1942, p. 11).

BOWERBANKIA GRACILIS

(Figure 8)

Bowerbankia gracilis is very common. It forms a soft grayish furry mass on 18 algal species. It consists of a number of transparent tubes clustered along a stolon, sometimes so densely that the stolon is scarcely visible. Caudal processes appear on some zooecia. The eight tentacles can be counted only when the animal is alive and in the expanded state. The zooids in Figure 8 are in the retracted state with the tentacles and gut (collectively called "polypide") withdrawn into the body cavity. Under such conditions the squared orifices show nicely.

BOWERBANKIA IMBRICATA

(Figures 9–10)

Bowerbankia imbricata was found on only five algal species by the authors. Additional species on which it has been reported are: *Ascophyllum nodosum* (Adams, 1800, p. 11), *Corallina officinalis* (Hincks, 1880, p. 521), *Cystoseira fibrosa*, *Fucus serratus* (Joliet, 1877, p. 294), *Desmarestia aculeata*, and *Furcellaria fastigiata* (Thompson, 1840, p. 252). Colonies may cover extensive areas of several inches, coating the "stems" and thalli of *Chondrus*. They do not exclude other forms from growing on the alga but may grow among hydroids, sponges, and other encrusting forms.

Superficially, dense growths of *Bowerbankia imbricata* and *B. gracilis* are indistinguishable. Imbricated colonies whose zooids were filled with large ciliated

PLATE III

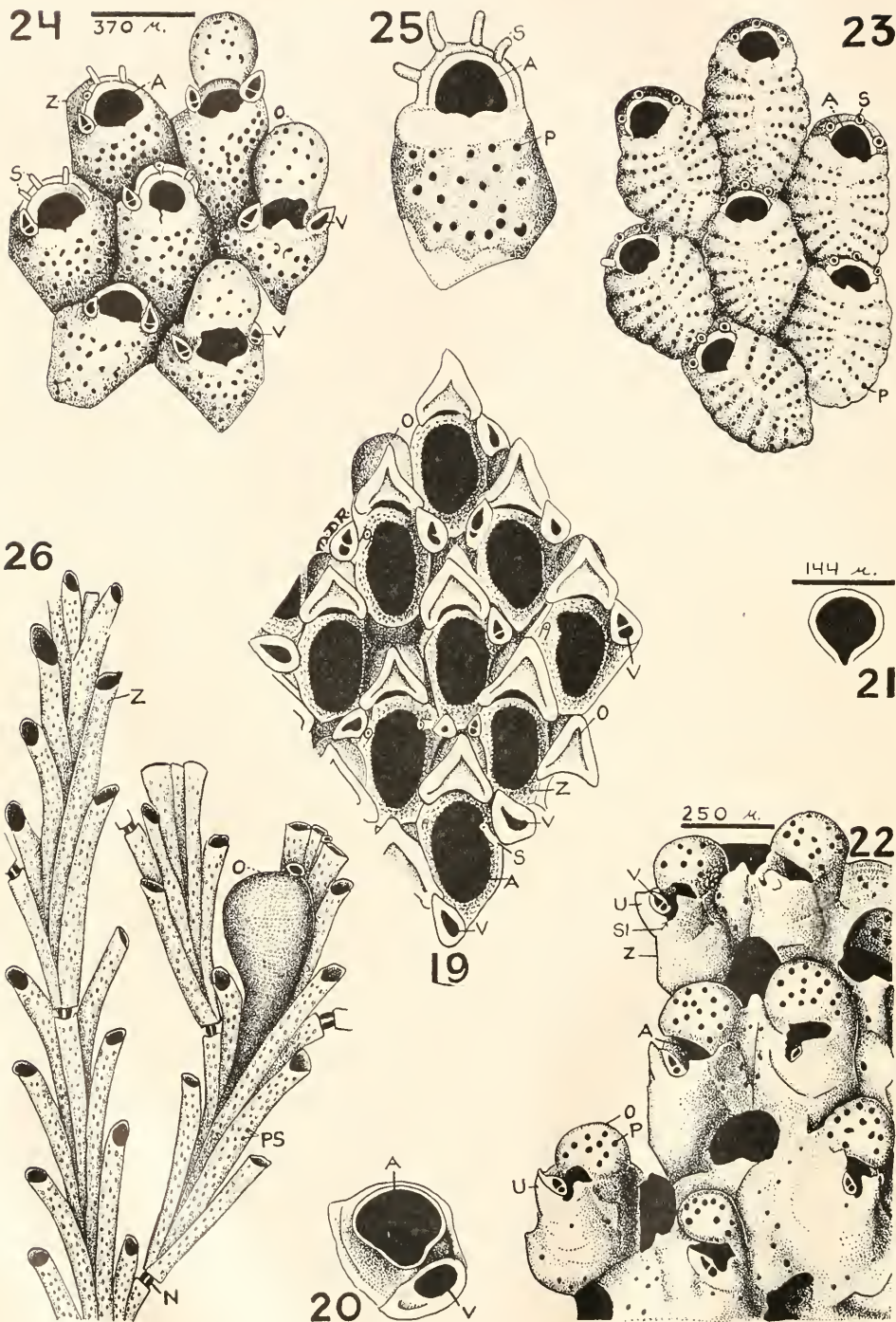
FIGURE 15. *Bugula flabellata*. The upper parts of three fertile ovicell-bearing zooecia (F) and an ordinary zooecium (R). Other structures shown are: (A) opesium, (O) ovicell, (S) spine, (V) avicularium. These same labels apply to the other figures on this plate.

FIGURE 16. *Bugula flabellata*. Broad flabellate branches with up to 6 rows of zooecia (Z) per branch. Some have ovicells, some avicularia, or both, and others have neither, at the moment. Drawn to the same scale as Figure 18.

FIGURE 17. *Bugula turrita*. Four fertile zooecia, each topped by a very shallow, fragile ovicell, set at an angle on the upper edge of the zooecium. Spines are well developed in this colony and one avicularium is shown. Drawn to the same scale as Figure 15. Collected at Woods Hole, VI–30–1938.

FIGURE 18. *Bugula turrita*. Branches showing biserial arrangement of zooecia (Z). Some zooecia bear ovicells (O).

PLATE IV



globular larvae were salmon pink in color because the red pigment of the larvae showed through the parent zoid walls. Such embryos were especially abundant in colonies collected during the first ten days in August (1947). Some embryos were found in colonies collected as late as August 31. Joliet (1877, p. 295) observed larvae during the month of July and reported that sexual reproduction took place from the end of June to early August.

Somewhat reniform larvae were released in great numbers on the morning of August 9, 1947. After a free-swimming period they attached to the substratum. Metamorphosis proceeded speedily, taking less than five minutes in some cases. The red color became concentrated at one end of the metamorphosing larva.

Adult zooids generally have ten tentacles and are square-topped when retracted. Measurements of seven retracted zooids were as follows: zoid length 0.925–1.374 mm.; zoid width 0.178–0.291 mm.; stolon diameter 0.040–0.101 mm.

Some very young colonies consisting of only one or two developing zooids had nine tentacles but their development could not be followed beyond a few days, so it could not be determined if these in time would increase their tentacular number to ten.

BUGULA CUCULLIFERA

(Figures 11–12)

Small fragments of this *Bugula* were found on *Fucus evanescens* and *Rhodomenia palmata* from Vineyard Sound on VIII-1-1945, on *Laminaria longicuris* from Provincetown, Mass., on VIII-18-1947 and on *Phyllophora membranifolia*, along with much *Crisia eburnea* and *Aetea sica* from New Bedford Harbor, on VIII-8-1945.

PLATE IV

FIGURE 19. *Callopora aurita*. Nine zooecia (Z) each capped by a rounded ovicell (O) which is decorated by a raised triangular ridge. Spines (S) and avicularia (V) are present near the large opesia (A). The same letters apply to the other figures on this plate. A calcined specimen from which all the soft tissues have been burned away. Drawn to the same scale as Figure 24.

FIGURE 20. *Cellepora dichotoma*. Part of a very lightly calcified specimen showing the shape of the aperture and the position of the avicularium. From Nobska Beach driftweed, Woods Hole, VII-25-1944. Drawn to the same scale as Figure 21.

FIGURE 21. *Cellepora dichotoma*. Aperture of a very young zooecium.

FIGURE 22. *Cellepora dichotoma*. Portion of a moderately calcified colony. An avicularium is borne on the side of the umbo (U) and faces toward a sinus (SI) in the peristome or raised shelf encircling the front of the aperture. The ovicells have pores (P).

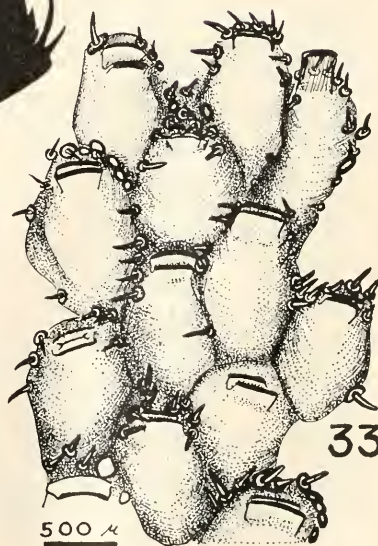
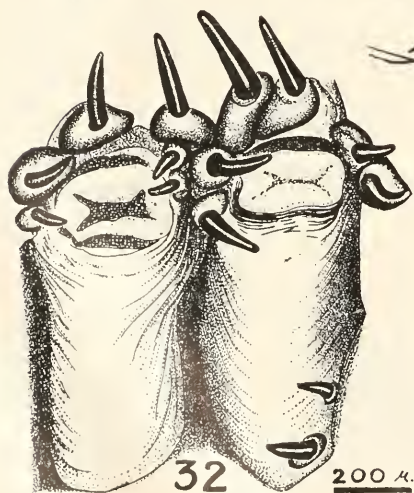
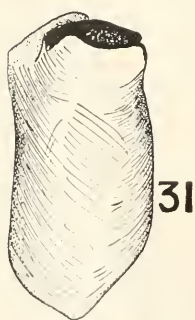
FIGURE 23. *Cribrilina annulata*. Seven zooecia. Drawn to the same scale as Figure 24.

FIGURE 24. *Cribrilina punctata*. Seven zooecia, three of which are capped by ovicells. One or two avicularia border the wide aperture. From Penikese Island, Mass., VIII-3-1947, on *Chondrus crispus*.

FIGURE 25. *Cribrilina punctata*. A more heavily calcified zooecium with 4 spines above the aperture. From Gay Head, Martha's Vineyard, VII-30-1946. Drawn to same scale as Figure 21.

FIGURE 26. *Crisia eburnea*. Four internodes, separated by dark yellow horny joints or nodes (N), bear a number of tubular autozooids (Z). One internode bears the greatly swollen ovicell or oöcium. The zooecia have numerous pseudopores (PS). From Black Rock, New Bedford Harbor, VIII-8-1945. Drawn to same scale as Figure 24.

PLATE V



Some embryo-filled ovicells were present. Very long rhizoid processes grew from the basal part of some of the colonies. Thirteen tentacles were counted on one zoid.

BUGULA FLABELLATA

(Figures 13, 15, 16)

A small colony of *B. flabellata* was found on *Fucus vesiculosus*. It was far less common than *B. turrita*. Also, it seemed to prefer attachment to piles, live cars, and other submerged wooden objects rather than to algae. It is a very sturdy form, growing in thick, fan-shaped, yellow-orange tufts (Fig. 13) which are about a half inch tall.

Glass slides submerged in Eel Pond at Woods Hole from August 13 to August 31, 1945, were heavily overgrown with various animal forms, including *Bugula flabellata*. Colonies of the latter were by then about $\frac{1}{4}$ inch tall and contained hundreds of zoids.

According to Grave (1933, p. 384) its breeding season is between June 1 and November 15.

BUGULA TURRITA

(Figures 14, 17, 18)

Bugula turrita is very common, growing on at least 16 algal species. In general appearance it is more plant-like than animal-like. It is of yellow-orange color and has a soft, fluffy, but firm texture. It has a beautifully spiralling manner of growth (Fig. 14). The colony branches into a number of spiralling "turrets." Some of the colonies may be $1\frac{1}{4}$ inches tall.

The tentacle number is about 14.

Ovicells were seen in colonies collected from the end of June through mid-August (Fig. 17). Many young colonies developed from released larvae during that time.

PLATE V

FIGURE 27. *Electra hastingsae*. Fifteen zooecia from the central part of a colony. One zooecium has lost all the spines around its opesium. The others have retained a varying number. Calcined specimen.

FIGURE 28. *Electra hastingsae*. A freehand sketch showing the flat, spray-like mode of growth which is so characteristic of this species. About natural size.

FIGURE 29. *Electra pilosa*, long-spined form. Tip of an alga, *Desmarestia aculeata*, completely encased by a bryozoan colony some of whose zooecia show an unusually long median spine. Shown in silhouette. Collected off Gay Head, Martha's Vineyard, VII-30-1946. Drawn to the same scale as Figure 33.

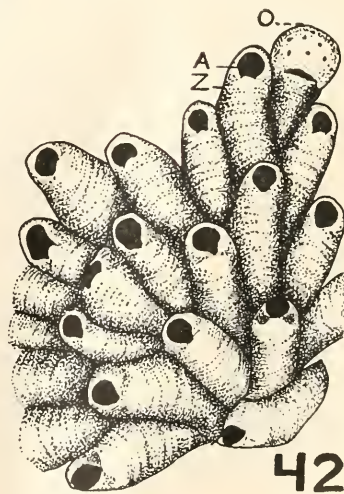
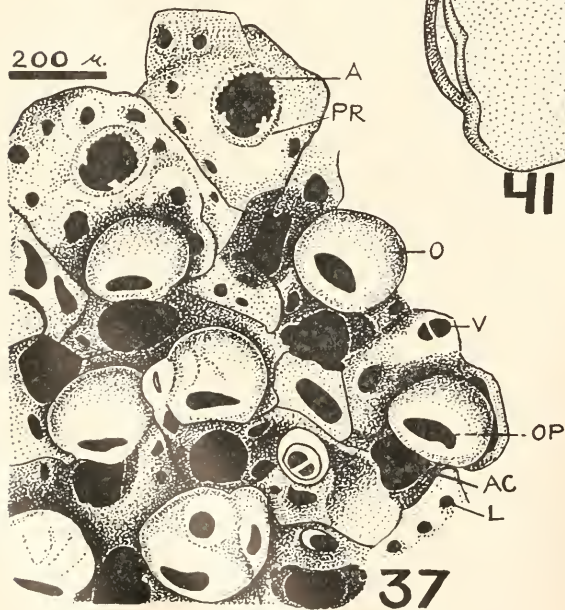
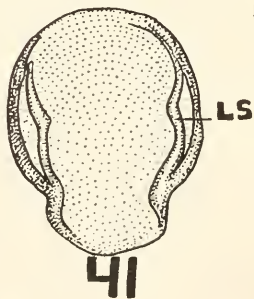
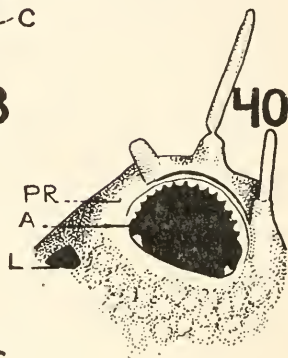
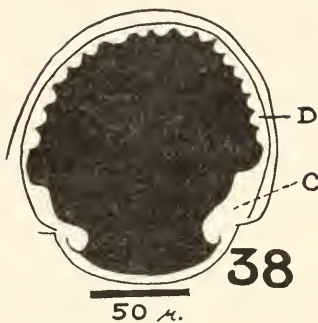
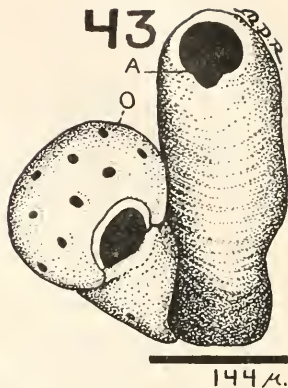
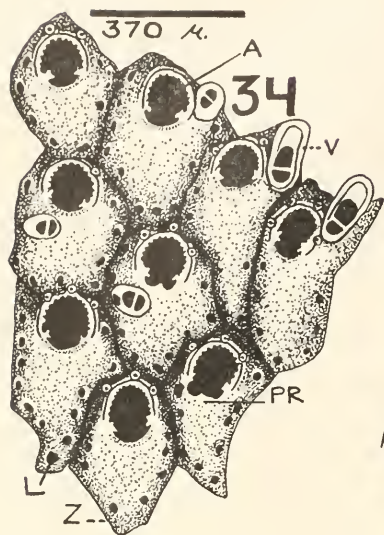
FIGURE 30. *Electra pilosa*, short-spined form. Four zooecia whose lowest, median spine is heavier and longer than the other opesial spines but not so long as the spines pictured in Figure 29. The two upper zoids show the crescent-shaped operculum rim in the upper part of the opesial area. The lower frontal wall of the zooecium is marked by numerous tremopores. From Devil's Foot, Woods Hole, VII-9-1945.

FIGURE 31. *Flustrella hispida*. A very young, spineless zoid. From Woods Hole, VIII-15-1939. Drawn to same scale as Figure 32.

FIGURE 32. *Flustrella hispida*. Two old zoids showing heavy "chitinization" of spines and lips of the orifice. The left zoid shows only circumnoral spines while the right shows those and also additional spines located lower down on the zoid. From same colony as Figure 31.

FIGURE 33. *Flustrella hispida*. Twelve spine-encircled zoids from a less heavily "chitinized" part of the same colony as Figure 32.

PLATE VI



CALLOPORA AURITA

(Figure 19)

Callopora aurita was not abundant on algal material, being found more commonly and in more extensive patches on rocks. Very small white colonies were found on specimens of *Phycodrys rubens* collected from Rye Ledge, Rye, New Hampshire, on IX-30-1945; on *Phyllophora membranifolia* dredged from Great Harbor, Woods Hole, Mass., on VIII-8-1946, and on holdfasts of *Laminaria Agardhii*. The colonies form a fine encrusting calcareous mesh on the algal thallus.

Ovicells were present in the colonies, but it was not possible to determine whether they were tenanted by larvae at the time of collection. Twelve tentacles were counted on one zoid.

CELLEPORA DICHOTOMA

(Figures 20-22)

Its small, white, calcareous zoaria grow on *Chondrus crispus*, *Gracilaria confervoides*, *Phyllophora Brodiaei* and *P. membranifolia*. Its zoids are crowded

PLATE VI

FIGURE 34. *Hippoporina contracta*. Portion of a young, uncrowded colony showing nine zooecia (Z), four of which are without avicularia (V) and three of which have a small rounded avicularium and two of which have spatulate avicularia (V). The distinctive serrate aperture (A) is readily distinguishable in these not heavily calcified zoids. The peristome (PR) is prolonged into a small bump or mucro below the aperture in the central zoid. Areolae (L) border each zooecium. The same labels apply to the other figures on this plate. Specimens dredged from Great Harbor, Woods Hole, on VIII-8-1946.

FIGURE 35. *Hippoporina contracta*. A small, slightly pointed avicularium with part of its aperture serrated. Drawn to same scale as Figure 43.

FIGURE 36. *Hippoporina contracta*. A small rounded avicularium with part of its aperture serrated. Drawn to same scale as Figure 43.

FIGURE 37. *Hippoporina contracta*. A more crowded and calcified colony than that of Figure 34. The upper two zoids show the typical serrate aperture. Seven ovicells (O) with large, comma-shaped pores (OP) are visible. Beneath each ovicell is a large peristome (AC) or peristomeal opening (here shown in black), at the bottom of which lies the distinctive serrate aperture (invisible in this picture). Calcined specimen.

FIGURE 38. *Hippoporina contracta*. The serrate aperture characteristic of this species. The aperture, black in this calcined specimen, in life is closed over by an operculum which is pictured in Figure 41. The aperture has 14 to 18 small rounded denticles (D) and two large bifid cardelles (C).

FIGURE 39. *Hippoporina contracta*. A spatulate avicularium seen at an angle. Drawn to the same scale as Figure 43.

FIGURE 40. *Hippoporina contracta*. Upper half of a zooecium showing areola (L), denticles, cardelles, peristome (PR) and three spines above the aperture (A). Drawn to same scale as Figure 43.

FIGURE 41. *Hippoporina contracta*. Operculum which closes the aperture of the zooecium. It has a stiffened rim and lateral sclerites (LS). Drawn to same scale as Figure 38.

FIGURE 42. *Hippothoa hyalina*. A colony showing a number of ordinary zooecia (Z) and a dwarfed one topped by an ovicell (O). Drawn to same scale as Figure 34.

FIGURE 43. *Hippothoa hyalina*. Another view of the punctate ovicell, its dwarfed zooecium and a normal sized zooecium. The latter shows the typical aperture, rounded and with a sinus. The transverse grooving normally found in the zooecia is faintly indicated in the larger zoid. Specimens dredged off Gay Head, Martha's Vineyard, VII-30-1946.

against each other. Embryo-filled ovicells were present at the time of collection (July 25, 1944).

There is some question as to the classification of this species. *Cellepora americana*, *Cellepora avicularis* and *Cellepora dichotoma* show such integradation that their exact status or validity needs critical review by some future worker. The species of the present study is identical with Marcus' illustration of *C. dichotoma* (Marcus, 1938, Plate XI, Fig. 26).

The species characteristics are as follows: (1) peristome with a sinus next to a raised umbo on the side of which is an avicularium facing the sinus; (2) aperture rounded, with postral sinus; (3) ovicell with pores, rounded and somewhat flattened; and (4) a few small pores (areolae) around the frontal wall of the zooecium.

A heavily calcified zoarium may show the ovicells almost completely immersed on all sides except the frontal in the secondarily calcified zooecial wall. The frontal of such ovicells is provided with good-sized pores and is at a lower level than the secondarily calcified outer zooecial wall.

CRIBRILINA ANNULATA

(Figure 23)

This encrusting species was very uncommon. Only one white calcareous zoarium was found on *Phycodrys rubens*, from Rye, N. H., on the reverse side of the thallus from the finer, more fragile *Cribrilina punctata*. Three or four spines were present around the aperture. Marcus (1940, p. 203) reported *C. annulata* from *Laminaria*.

CRIBRILINA PUNCTATA

(Figures 24-25)

Small patches of this fragile white calcareous form were found encrusting seven algal species. The number of spines around the aperture varied from none to five. The frontal pores were somewhat irregular in size and position. This was not a very common form; only a few colonies appeared in the collection.

CRISIA EBURNEA

(Figure 26)

Crisia eburnea was exceedingly common on 18 algal species. It was especially abundant on *Chondrus crispus*, the two *Phyllophorae*, and *Phycodrys rubens*. A very large amount of it was collected from the driftweed along the beaches at Nobska, Gay Head, and Cuttyhunk. Dried specimens were just as useful as wet ones for taxonomic purposes.

Prenant and Teissier (1924, p. 18) reported *C. eburnea* from *Halidrys* and certain *Cystoseiras*.

It forms brittle, white, openly dendritic tufts up to 7 mm. tall on the thalli of the small, and around the holdfasts of the large, algae. A *Crisia* colony consists of a number of calcareous tubular zooecia forming internodes which are separated

from other internodes by short, narrow, yellowish to brown chitinous joints. A branch which consists of ordinary tubular zooecia (autozooids) alone is called a sterile internode. Three such are pictured in Figure 26. A branch which consists of a number of autozooids and a long, very swollen brood chamber (ooecium or ovicell) is called a fertile internode. One is pictured in Figure 26. In the identification of different species of Crisiidae the number of zooecia in the fertile and sterile internodes is important. In *Crisia eburnea* the sterile internode has four to eleven zooecia, and a fertile internode seven to ten (Borg, 1944, p. 158).

Ovicells were found on specimens collected throughout the summer months. Embryos were seen in some on August 8, 1946.

CRYPTOSULA PALLASIANA

Cryptosula pallasiana forms a round, flat, regularly patterned, pale orange to white encrustation on rocks, shells, and algae. It occurs more commonly and forms larger colonies on the harder substrates than on the algae but is not uncommon on the latter. It was found on eleven algal species which came from a number of collecting sites between Martha's Vineyard, Woods Hole, North Falmouth, and New Bedford (all in Massachusetts). They grew on the thalli of algae and on the *Laminaria* holdfasts. Colonies attached to *Enteromorpha intestinalis* and *Ulva Lactuca* var. *rigida* were young and small, consisting of few (five or less) freshly formed zooids (as of VIII-13-1945). Submerged glass slides, left in Eel Pond at Woods Hole for the first two weeks in July and kept a week longer in running sea water in the laboratory, were well covered with many animal forms including *Bugula turrita*, *Pedicellina cernua* and *Cryptosula pallasiana*. The *Cryptosula* colonies had from one to thirty zooids on these slides. Their polypides had 16 tentacles.

Barrois (1877, p. 139) reported larvae in August and September. The Woods Hole specimens produced larvae in those months as well as during June and July.

Joliet (1877, p. 291) reported this bryozoan on *Callothrix pannorum*. Prenant and Teissier (1924, p. 23) reported *Cryptosula* from other *Laminariae*, *Himantalia*, and *Saccorhiza bulbosa*.

No drawings of *Cryptosula* are here included because the species was previously figured (Rogick, 1945b, p. 3, Fig. 1).

ELECTRA HASTINGSÆ

(Figures 27-28)

A few small colonies of *E. hastingæ* encrusted the thalli of *Fucus vesiculosus* var. *spiralis* and *Laminaria Agardhii*. Marcus (1938, p. 17) reported the bryozoan from *Zostera*. Sometimes it grows on the gill chamber of *Libinia* crabs. Generally, however, the bryozoan is found on hard substrates (rocks and shells) more often than on algae.

Electra hastingæ is a fragile, white, calcareous species, forming completely adherent frond-like traceries on the substratum (Fig. 28). Some colonies lack spines around the opesium. Other colonies have a variable number of very delicate ones, sometimes as many as 18. Some of the spines may break off (Fig. 27).

A new zooecium may occasionally grow right out of the opesium of another empty one. Whether that is a case of regeneration or the settling of a new larva on an old colony, is not certain. Embryos were not observed.

ELECTRA PILOSA

(Figures 29-30)

Electra pilosa is an extremely common calcareous but fragile encrustation on 17 algal species. It has been reported previously from: *Fucus serratus* (Joliet, 1877, p. 290); *Ulva* (Hutchins, 1945, p. 540); *Laminaria saccharina* (Leidy, 1855, p. 9); *Furcellaria* and *Polyides* (Marcus, 1940, p. 118); the *Cystoseiras*, *Corallina* (Prenant, 1927, p. 24) and *Zostera* (Prenant, 1932, p. 92).

Electra pilosa forms grayish-white, single-layered colonies which spread like a fine, closely-woven mesh over large areas, sometimes a foot in length, of algal thalli. *Laminaria* and *Rhodymenia* thalli are particularly favored. Numerous colonies may grow toward and into each other to form an almost continuous thin crust over the thalli. The lacy *Plumaria sericea* fronds, in some instances, were completely encased in *Electra pilosa*. Many Foraminifera were scattered over the *Electra*.

Great variation in degree of spination occurs. Several *E. pilosa* "forms" of dubious validity are mentioned in literature: forma *typica*, f. *dentata*, f. *laxa* and f. *verticillata*, differing slightly from each other, mainly in the presence or length of the principal median proximal spine. Borg (1930, p. 63) and others mentioned that occasionally several of these growth forms may be found in a single *E. pilosa* colony, and therefore should not be considered valid varieties.

The present writers found both long-spined (forma *verticillata*, Fig. 29) and short-spined (forma *dentata*, Fig. 30) growths in the collections, the latter being far more common than the long-spined specimens.

Tentacles numbered 12 to 14.

FLUSTRELLA HISPIDA

(Figures 31-33)

Flustrella hispida grows on five Woods Hole algal species: *Ascophyllum nodosum*, *Chondrus crispus*, *Fucus vesiculosus*, *Phyllophora membranifolia* and *Ulva Lactuca* var. *rigida*. Also, the M. B. L. Collecting Crew has on numerous occasions brought in *Ascophyllum* covered with *Flustrella* from other localities. Additional algae from which it has been recorded are: *Gigantina mamillosa* (Hincks, 1880, vol. 1, p. 507); *Fucus serratus* and *Cystoseira* (Joliet, 1877, p. 292). It was far more common on *Ascophyllum* and *Fucus* than on the green or red algae in the Woods Hole region.

Flustrella hispida forms a brown, rubbery, and somewhat slimy crust over extensive areas of the algal thallus. The zooids are fairly soft and baggy (Figs. 31, 32). Thirteen tentacles were counted on one specimen. Spines were lacking in the very youngest zooids (Fig. 31), but more mature ones show variation in distribution and number of spines (Figs. 32, 33). In the oldest parts of the colony the spines may become very thick and dark reddish brown, and appear mounted on horny pads (Fig. 32) about the zooeial orifices. In younger colonies, and also in some older zoecia, as in the right zooid of Figure 32, spines appear elsewhere about the zooid than just around the orifice. The reinforced orifices are shaped like the top of a purse (Figs. 32, 33).

Barrois (1877, p. 214) found *F. hispida* colonies filled with embryos during the months of May, June and July.

HIPPOPORINA CONTRACTA

(Figures 34-41)

White to buff-colored colonies of this species were found more often on rocks and shells than on algae. However, some did grow on *Phyllophora Brodiaei* and *P. membranifolia*, and were up to 2 cm. in diameter. The appearance of the colony varies greatly, depending upon the age of the colony, degree of calcification, the presence of ovicells and the nature of the substratum (compare Figs. 34 and 37).

The key character in identifying this species is the "beaded" aperture (Fig. 38) whose circular outline, serrate antral border, and two bifid cardelles marking the postral border vary so little that they can be identified in either old or young colonies. The number of rounded denticles in the antral border ranges from 14 to 18. In old, heavily calcified colonies (Fig. 37), the zooecial wall and peristome around the aperture may increase in thickness so greatly that the primary "beaded" aperture comes to lie considerably below the external body wall surface, at the bottom of a calcareous "well," the wall of which is formed by the peristome. The top opening of this calcareous "well" is called either the secondary aperture or the peristomice (Fig. 37, AC).

In younger, less calcified colonies, two to six oral spines, sometimes measuring 0.12-0.13 mm., may appear on the peristome (Fig. 40). These break off and their bases may become completely overgrown in the process of increasing calcification of the body wall.

Marcus reported 12 tentacles for this species (1937, p. 98).

The ovicells are quite characteristic also. They are smooth, hemispherical, and provided with a large, comma-shaped membranous area or pore (Fig. 37, OP) on the frontal surface.

Six to thirteen marginal pores or areolae (Fig. 34, L) can be seen in the zooecial body wall.

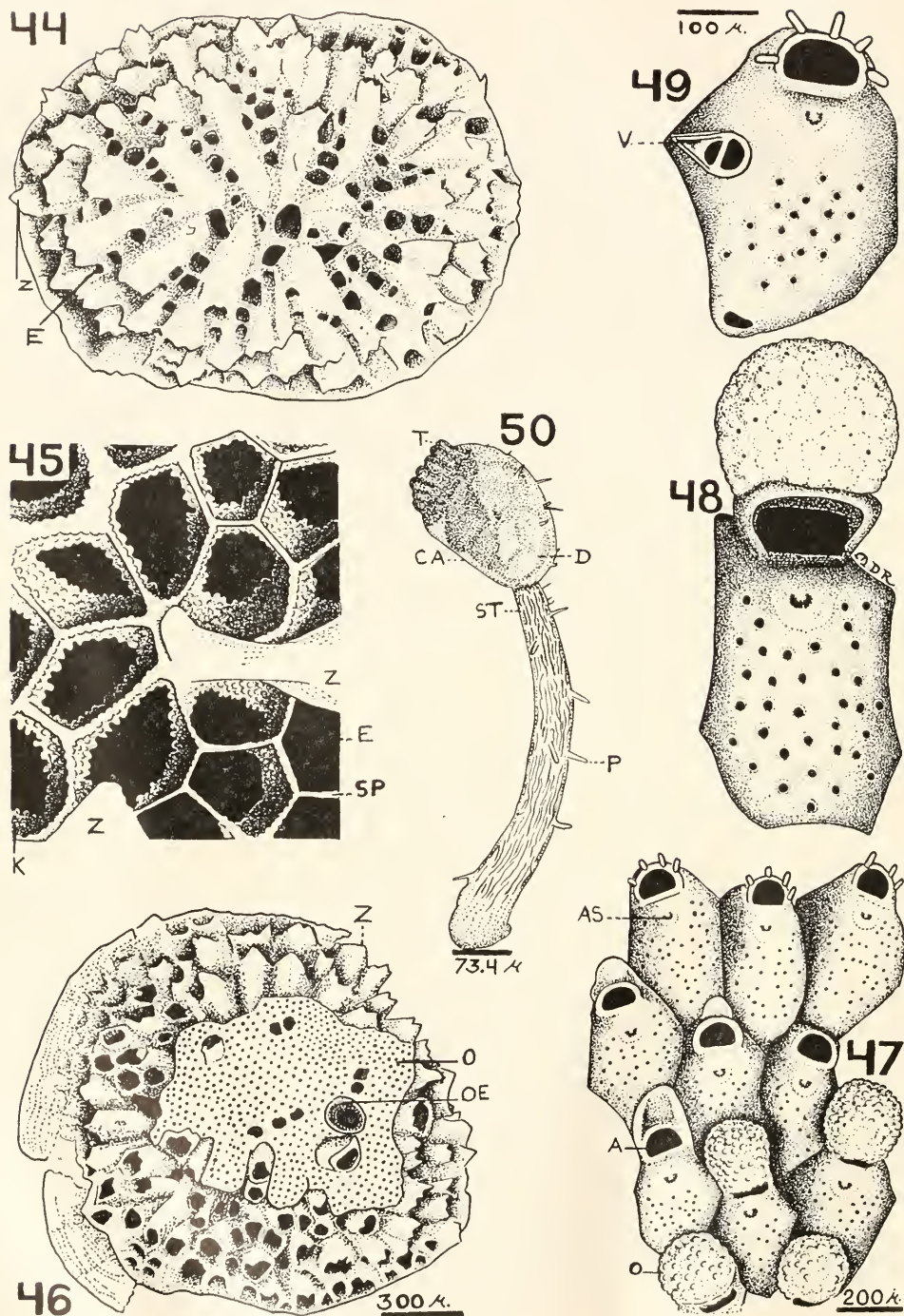
HIPPOTHOA HYALINA

(Figures 42-43)

Hippothoa hyalina was extremely common on 17 algal species in the Woods Hole region. Borg (1930, p. 84) listed it from *Laminaria saccharina*; Prenant and Teissier (1924, p. 22) from the Florideae and Dictyota, and Prenant (1927, pp. 26-27) from *Laminaria cloustoni* and *Saccorhiza bulbosa*. A dry herbarium mount of a pressed *Phycodrys rubens* (collected by Dudley at Marble Head in Sept. 1874) was examined by the writers and found to contain easily recognizable and uncrushed *H. hyalina* zoaria.

Hippothoa hyalina forms glistening white or grey calcareous patches usually from 1 to 8 mm. in diameter, either on or encircling the thalli of most of the mentioned algae and on the holdfasts of *Laminaria* and *Rhodomenia*. "Stems" or filaments of *Cystoclonium purpuraceum* var. *cirrhosum* were encased in rough

PLATE VII



calcareous sheaths of *H. hyalina* sometimes an inch in length. Often the sheaths of colonies were arranged in a linear series, the total series attaining a length of several inches.

Embryo-filled ovicells were plentiful in specimens collected during July and August in the Woods Hole area. Many ancestrulae or the single individuals from which a colony begins were observed in collections made up to August 11, 1945. These ancestrulae arise from sexually produced larvae. Barrois (1877, p. 164) remarked that at Roskoff the embryos were carried in transparent ovicells in the months of May and June, so apparently the breeding season is of considerable length.

LICHENOPORA HISPIDA

(Figures 44-46)

One fertile and less than a dozen small immature colonies of this species were found growing on *Phycodrys rubens* and *Phyllophora membranifolia* collected from Rye, N.H. on IX-30-1945.

The fertile colony (Fig. 46) has a brood chamber provided with a thin, rounded aperture and many small pores. The autozooids (Fig. 44) terminate in jagged edges. They are partly surrounded by reticulate alveoli (Fig. 45) which are lined with small calcareous projections from the interalveolar septa whose thickness is variable.

Borg (1926) gives a good account of the development of various Cyclostomata, including the Lichenopora, and discusses the terminology of the group.

MEMBRANIPORA LACROIXII (?)

Membranipora lacroixii is a species whose identification and synonymy are exasperatingly confused in literature. Part of this is due to vague original

PLATE VII

FIGURE 44. *Lichenopora hispida*. A fairly young colony showing autozoecia (Z) separated by large cavities or alveoli (E). Drawn to same scale as Figure 46.

FIGURE 45. *Lichenopora hispida*. Detail of the center of an immature though fair-sized colony, showing about 17 alveolar spaces (E), the interalveolar septa (SP) between them and the projections (K) from the calcareous cryptocyst of the septal wall. The sides of two autozooids (Z). Drawn to same scale as Figure 49.

FIGURE 46. *Lichenopora hispida*. A damaged, fertile colony, showing an irregular, punctate brood chamber (O), the brood chamber aperture (OE) and numerous short (immature or damaged?) and some normal autozooids (Z).

FIGURE 47. *Microporella ciliata*. Nine zooecia, four of which have well-developed ovicells (O) and three of which have shelf-like beginnings of ovicells distal to the aperture (A). Each zooecium has a crescent-shaped ascopore (AS) and smaller frontal pores. Oral spines occur on the upper three zooids. The crescent-shaped ascopore and the hemispherical aperture are key characters for this species.

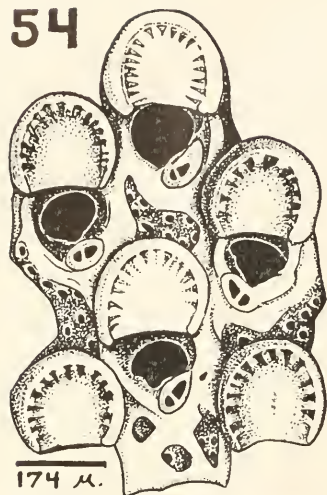
FIGURE 48. *Microporella ciliata*. A zooecium topped by an ovicell. Both have pores but of different size. Drawn to same scale as Figure 49.

FIGURE 49. *Microporella ciliata*. A zooecium showing oral spines and a pointed avicularium (V) in a characteristic position. The avicularium may develop on either the right or left side of the zooid. Same collection area and date as Figure 43.

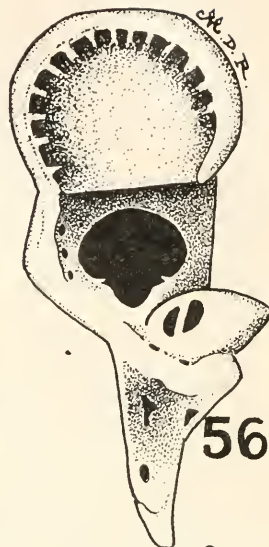
FIGURE 50. *Pedicellina cernua*. A single zooid consisting of a stalk (ST) and a calyx (CA) containing the polypide (D) and rolled in tentacles (T). A few spines (P) occur on the stalk and calyx. From Black Rock, New Bedford Harbor, VIII-8-1945.

PLATE VIII

54



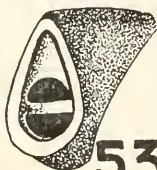
55



56



52



53



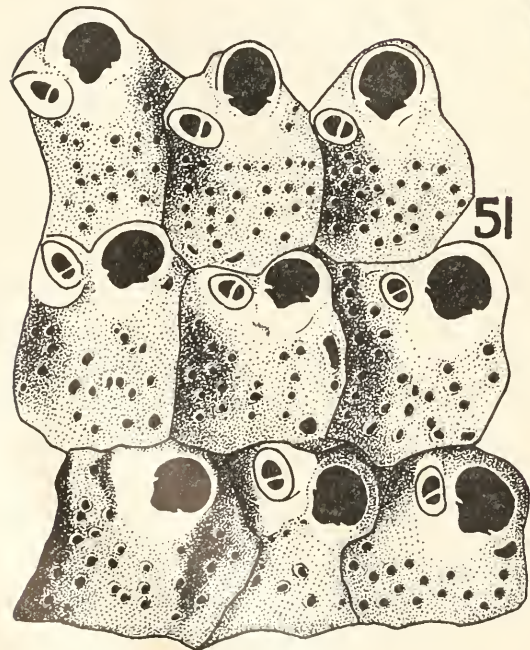
60



59



57



51



58

148 μ.

descriptions, and part due to the apparently great variation in spination and degree of calcification of the zooecia. Such will continue to be the state of affairs until someone takes the trouble to make a very elaborate study of the variations of this species. The Woods Hole and New Rochelle specimens of the present study resemble the *Membranipora lacroixii* pictured by Osburn (1912, Plate 22, Fig. 22), the *Conopeum lacroixii* pictured by Canu and Bassler (1920, Plate 13, Fig. 9), the *Conopeum reticulum* pictured by Harmer (1926, Plate 13, Fig. 12), the *Biflustra aciculata* of MacGillivray (1891, Plate 9, Fig. 5) and the *Membranipora crustulenta* of Osburn (1944, Fig. 20, p. 32). Our specimens differ from the *Conopeum reticulum* pictured by Marcus (1938, Plate 2, Fig. 5A), the *Conopeum lacroixii* figured by Canu and Bassler (1923, Plate 29, Fig. 4), the *Membranipora reticulum* f. *lacroixii* and *M. crustulenta* of Borg (1930, pp. 63-65). The present study specimens definitely are not the *M. crustulenta* of Borg because that species is pictured with a calcified operculum, a character not present in our specimens. Until the status and limits of the species are fixed, the present authors will continue to call it *M. lacroixii*, as in Osburn's 1912 paper.

Membranipora lacroixii was found encrusting rocks, shells, and less frequently the algae *Ascophyllum Mackaii*, *Chondrus crispus* and *Phyllophora membranifolia*. It formed a delicate, gray-white tracery which adhered so closely to the substratum, especially rocks, that it was difficult to dislodge. No avicularia or ovicells were found. The conspicuous triangular spaces mentioned as characteristic by Harmer and Marcus were not observed on our specimens. Calcifica-

PLATE VIII

FIGURE 51. *Schizoporella biaperta*. Nine regularly arranged, moderately calcified zooecia, one of which is without an avicularium. Drawn to the same scale as Figure 54. Calcined specimen.

FIGURE 52. *Schizoporella biaperta*. A small ellipsoidal avicularium. Drawn to same scale as Figure 55.

FIGURE 53. *Schizoporella biaperta*. A small oval or somewhat pointed avicularium. Drawn to same scale as Figure 55.

FIGURE 54. *Schizoporella biaperta*. A fertile area of a colony showing six ovicells. The middle ovicell is most nearly typical in appearance. The frontal area of its zooecium is more highly calcified than that of the other three zooecia above it, and than that of the zooecia of Figure 51.

FIGURE 55. *Schizoporella biaperta*. A moderately calcified zooecium showing two avicularia, the two apertural teeth (cardelles) and the sinus between them. A typical specimen.

FIGURE 56. *Schizoporella biaperta*. A heavily calcified zooecium topped by an ovicell. The zooecial shape is atypical and due to crowding in the colony and to excessive calcification. The avicularium is heavily calcified. The depressed rim of the ovicell frontal has been accidentally over-emphasized and should look less depressed (see Figure 54, middle ovicell). Drawn to same scale as Figure 55.

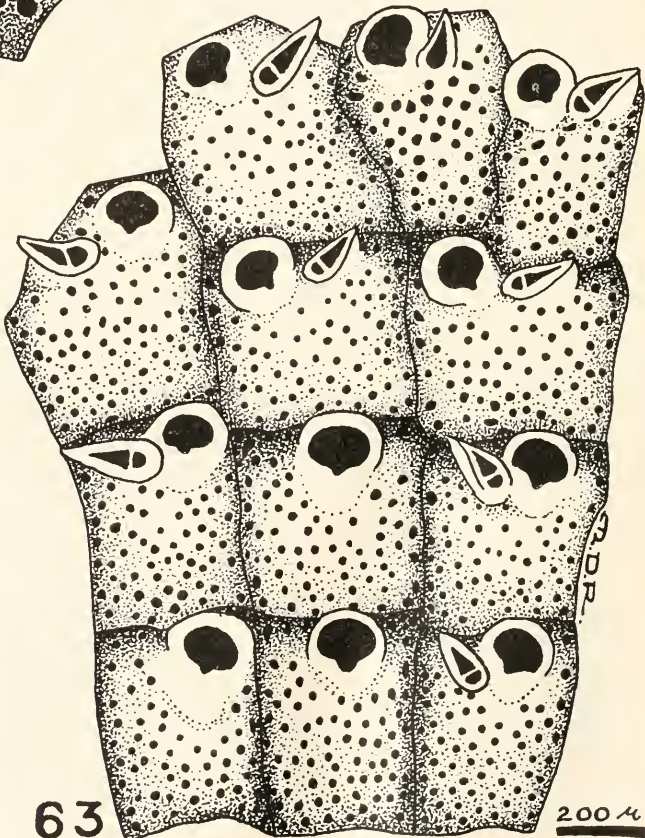
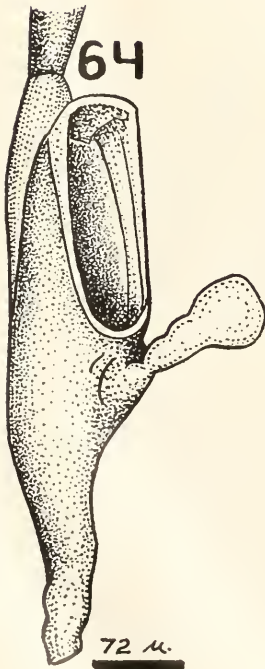
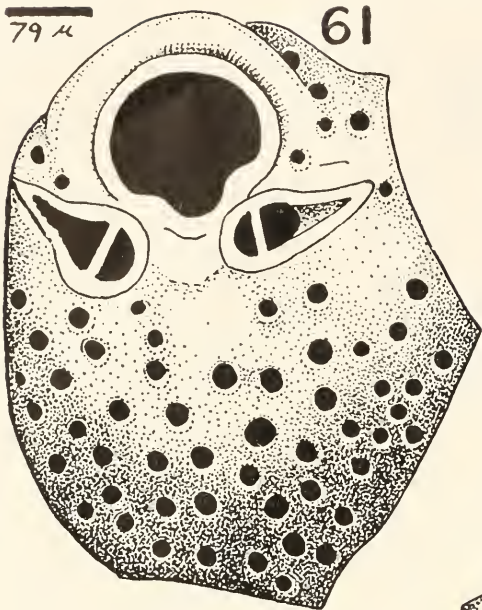
FIGURE 57. *Schizoporella unicornis*. A sharply pointed avicularium. Drawn to same scale as Figure 55.

FIGURE 58. *Schizoporella unicornis*. Three zooecia, two of which have ovicells. The bottom zooecium is twice as broad as the other two to which it gives rise. The zooecial frontal wall and the ovicells have pores. The sharply pointed avicularia vary in size and are near the aperture.

FIGURE 59. *Schizoporella unicornis*. Another unusually shaped and very broad zooecium which would give rise to two rows of zooids. The pores of its frontal area and of the ovicell are better shown. Drawn to the same scale as Figure 54.

FIGURE 60. *Schizoporella unicornis*. A smaller pointed avicularium, drawn to the same scale as Figures 55 and 57.

PLATE IX



tion was not heavy. Some New Rochelle specimens had up to ten spines, while others had no spines around the aperture—all in the same colony. Some zooids had 11 tentacles.

This species was pictured in an earlier paper (Rogick, 1940, p. 167, Figs. 6–9).

MEMBRANIPORA TUBERCULATA

A specimen of *Membranipora tuberculata* was found on fronds of *Cryptopleura* sp. and *Sargassum* sp. which were sent to the writers by Dr. William Randolph Taylor. The *Cryptopleura* had come from Puerto de la Paloma, Uruguay, from the collection of Carmen de Franco de Pimienta. The *Sargassum* sp. had been collected by Adrien Questel on April 21, 1944, from Guadeloupe, Antilles. This *Membranipora* has been previously reported by Marcus from *Laminaria* (1939, p. 126) and Fucus (1937, p. 34); by Hastings (1929, p. 706) from *Padina*; and by Osburn (1912, p. 231) from *Sargassum bacciferum* which had drifted into Vineyard Sound.

The extensive colonies of *M. tuberculata* spread flatly over the algal fronds in an ivory-white lacework, reminiscent of *Electra pilosa*. The two or three prominent calcareous tubercles at the anterior end of each zooecium from which this species gets its name may project separately and distally or may coalesce, forming a somewhat rounded ledge.

Since *M. tuberculata* was adequately pictured in both Osburn's (1912, as *M. tchuelca*) and Marcus' (1937) papers, no figure of it was included in the present study.

MICROPORELLA CILIATA

(Figures 47–49)

Small, flat, circular colonies, white to iridescent in color, calcareous though fragile, encrust shells, rocks, and five algal species in the Woods Hole region. Prenant and Teissier (1924, p. 23) found *Microporella ciliata* on three additional algae: *Himanthalia*, *Laminaria saccharina* and *Saccorhiza bulbosa*. Hadley Harbor specimens were found growing on the same thallus with *Foraminifera*, *Aetca sica*, *Crisia eburnea*, *Hippothoa hyalina*, and *Schizoporella biaperta*.

PLATE IX

FIGURE 61. *Schizoporella unicornis*. A single zoid showing typical rounded aperture with its postral sinus and two pointed avicularia situated on either side of the aperture. The presence of two avicularia is a less frequent condition than the presence of one avicularium. Frontal surface of zooecium has a number of closely set pores. Calcined specimen.

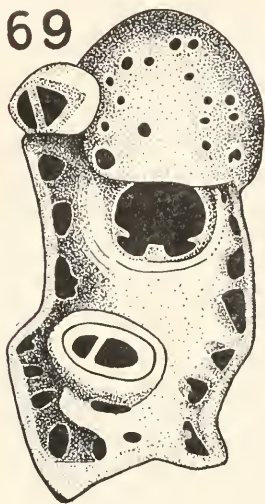
FIGURE 62. *Schizoporella unicornis*. Three zooecia showing varying degrees of calcification. The uppermost square zooecium has a completely calcified aperture. The second squared zooecium has the aperture and avicularium openings completely calcified or plugged up. Around the right, left and lower sides of this second zoid are white septa, outgrowths from a newly overgrowing colony whose marginal zooecium is shown as the partial, third, bottom zooecium with black aperture.

FIGURE 63. *Schizoporella unicornis*. Portion of a typical colony. Three rows of zooecia are at the bottom and four at the top of this colony fragment, showing how a colony may increase in width at the periphery. Five zooecia have the avicularium on one side of the aperture, four on the other and three are without avicularia. Apertures may be placed either in the middle or at one side of the distal part of the frontal surface. The frontal surface is rather flat in this colony. Calcined specimen.

FIGURE 64. *Scruparia ambigua*. A zoid with a frontal and distal bud. The basal proximal part of the frontal bud and of the zoid is slightly twisted, a typical condition.

PLATE X

69

79 μ .

66

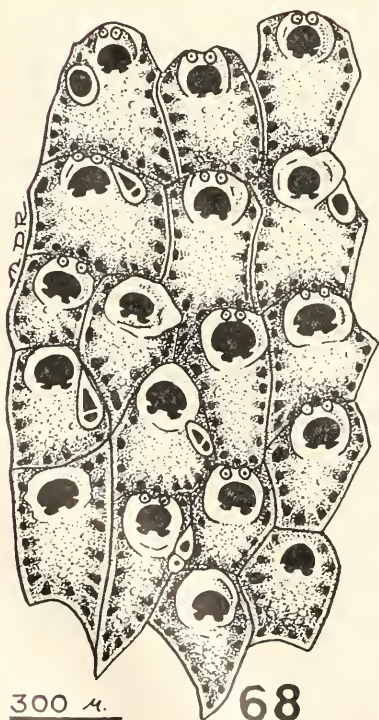
67

72 μ .

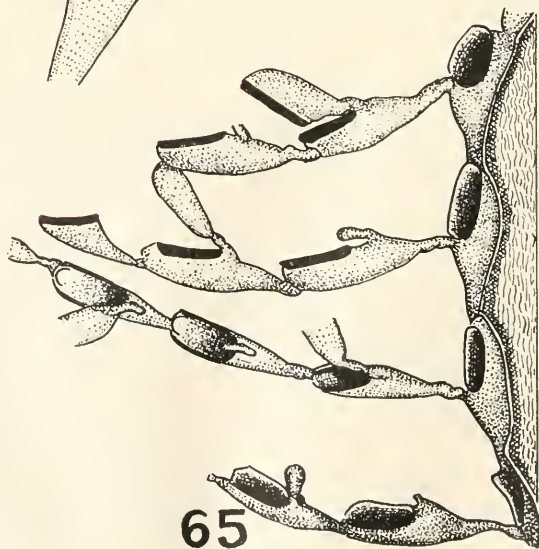
70



71

300 μ .

68



65

Some zooecia are without oral spines, avicularia or ovicells. Others have them. Oral spines may number from 3 to 7 (Figs. 47, 49). Ovicells are globose, "pebbled" in texture, and provided with small pores (Fig. 48). One avicularium is placed at an angle on either the right or the left frontal side, one-third to one-half of the way down and laterad from the aperture. The aperture is hemispherical and placed above the small crescent-shaped ascopore (Fig. 49). Canu and Bassler (1930, p. 47) reported 13 to 14 tentacles for this species.

Embryo-filled ovicells were collected on VIII-28-1939. Many very young colonies were found developing at that time also.

PEDICELLINA CERNUA

(Figure 50)

Pedicellina cernua though small, soft-bodied, and inconspicuous was reported on 11 algal species. Also, Leidy (1855, p. 11) reported it on the "roots" of *Laminaria saccharina*; Prenant and Teissier (1924, p. 19) reported it on the *Cystoseiras*, *Florideae* and *Dictyota*; and Joliet (1877, p. 296) on *Corallina squammata* and *Cladophora rupestris*.

It has a creeping stolon from which arise flexible stalked zooids. Spines occurred on the calyx and stalk (Fig. 50) of a few zooids but most specimens were without them. The tentacle number in several very young zooids was 8 to 12. The number increases with age. Marcus (1939, p. 212) gives the tentacle range for this species as 8 to 24.

PLATE X

FIGURE 65. *Scruparia ambigua*. A row of four zooecia encrusting a thick algal filament. From these arise four branches of zooids. The opesia rim of all but possibly the terminal budless individuals is parallel to the back of its own zooid. Drawn to the same scale as Figure 68.

FIGURE 66. *Scruparia clavata*. Two full-grown zooecia and two developing buds. The smaller, left bud belongs to a developing ovicell. The ovicelled individual originates below the other bud. Drawn to the scale above.

FIGURE 67. *Scruparia clavata*. Two grown zooecia and a third smaller one topped by a globose ovicell which has several large pores. The ovicell faces in the opposite direction from the ordinary zooecium. Drawn to same scale as Figure 66.

FIGURE 68. *Smittina trispinosa*. Eighteen ordinary zooecia growing in a very regular fashion. Some zooecia have rounded and some pointed avicularia while others have none at all. The flared peristome about the aperture is well developed in many. Some have spines, others do not. Calcined specimen.

FIGURE 69. *Smittina trispinosa*. A zooecium, ovicell and two avicularia. The circular aperture with the two cardelles (lateral teeth) and the lyrula (broader, median tooth) is a key character. So are the ovicell with its pores and leaning avicularium and the row of marginal zooecial areolae. Avicularia may be found in various locations on the frontal zooecial surface (compare this figure with Figures 68 and 71). The zooecium shows secondary calcification about the lower avicularium and partly covering the lower areolae. Drawn to same scale as Figure 70. Calcined specimen.

FIGURE 70. *Smittina trispinosa*. An ovicell topped by a triangular avicularium. The zooecial peristome is heavily calcified and forms a collar at the bottom of which can be seen the aperture, lyrula and cardelle.

FIGURE 71. *Smittina trispinosa*. A young zooid showing the characteristic apertural features and a frontal triangular or pointed avicularium. The frontal surface is slightly "beaded" and marginal areolae outline the thin edge. Drawn to same scale as Figure 70.

SCHIZOPORELLA BIAPERTA

(Figures 51-56)

This white to reddish-orange bryozoan is fairly common on nine algal species. Its colonies attain a diameter of 2 cm. or more and appear fairly sturdy. They grow either flat on the thallus or may extend beyond the thallus, forming calcareous "ruffles" which may be lamellate (several layers in thickness).

The key characters of this species are: (1) rounded aperture with a postoral sinus between the two cardelles (Fig. 55); (2) one or two small oval or ellipsoidal avicularia (Figs. 52, 53) mounted on mammillate prominences at the right or left or both sides of the sinus area (Figs. 51, 55); (3) frontal wall perforated by irregularly sized and spaced pores, and (4) hemispherical ovicells the edge of whose frontal area is slightly depressed and marked by faint calcareous ribs (Figs. 54, 56). Heavy calcification obscures some of these characters, especially the porous frontal area of the zooecium (Figs. 54, 56).

Twelve tentacles were counted on one zoid.

In July and August, the ovicells contained red embryos or larvae.

SCHIZOPORELLA UNICORNIS

(Figures 57-63)

Schizoporella unicornis is very common on rocks and shells, but less frequent on algae. It grew on six Woods Hole region algal species. Additional algal hosts mentioned by Prenant and Teissier (1924, p. 23) are the Florideae, Himanthalia, and *Saccorhiza bulbosa*.

There is great variation in the appearance of the colonies. Their color ranges from white to reddish orange to a dull red. Some are smooth, flat, and shining, others rough and extended beyond the thalli. They may be lamellate, one colony growing over another. No description of the species is necessary because Figures 58 and 59 show the ovicells, and Figures 61 and 63 show the typical zooecial appearance, growth habit, aperture shape, and disposition of the avicularia. The avicularia grade in size (Figs. 57-60), but are always sharply pointed. Calcification may sometimes obliterate them and the aperture (Fig. 62).

In the Woods Hole area, larvae were found in ovicells in July and August (the times when collection was made) and undoubtedly occurred before and beyond these dates. At Beaufort, North Carolina, they are found the year round, according to McDougall. McDougall (1943, p. 340) observed that the times of greatest abundance of larvae (as judged by settlements on experimental substrata there at Beaufort) were in May, June, September, October and November.

SCRUPARIA AMBIGUA

(Figures 64-65)

Most of the specimens of this dainty little dendritic form were dredged off Gay Head, Martha's Vineyard, on VII-30-1946. Some were growing on *Bugula turrita*, others on eleven algal species. It also grew in close association with hydroids and *Hippothoa hyalina*.

Scruparia ambigua zoids are yellowish, horny, transparent, and slender. They ranged from 0.345 to 0.495 mm. in length, the average of 12 specimens being 0.431 mm. The branching of the colony is quite open. Tentacles numbered ten in each of two zoids. No ovicells or larvae were found in the present material. Barrois (1877, p. 194) found ovicells and larvae at Roskoff during the month of June.

Hastings (1941) made a careful study of this species and differentiated *Scruparia ambigua* from *S. chelata* on the basis of the opesial slant and encrusting zooecia. In *S. ambigua* the opesial rim is parallel to the basal wall of the zooecium, and the free zooecial branches arise from a series of encrusting zooecia, as in Figure 65.

SCRUPARIA CLAVATA

(Figures 66-67)

A few scraps of this delicate, horny, dendritic, transparent bryozoan were growing on *Laminaria Agardhii* which was dredged off Gay Head, Martha's Vineyard on VII-30-1946. Some ovicells were present. The zooecia bearing them were slightly smaller (Fig. 67) than the other zooecia. The zooecial orifice is much smaller than that of *Scruparia ambigua*.

Marcus (1940, p. 208) created a new genus *Haplota* for *S. clavata*.

SMITTINA TRISPINOSA

(Figures 68-71)

This species was found with great frequency on shells and rocks, sometimes many layers in thickness on the latter. However, its occurrence on algae was infrequent. *Chondrus crispus* (from North Falmouth, Mass.), *Laminaria Agardhii*, *Phyllophora Brodiaei*, and *P. membranifolia* from Woods Hole had a few colonies.

Rock colonies or nodules often are a light mustard yellow color; colonies on algae, however, were never that striking a color, but were ivory or iridescent.

Colonies are very fine grained in general appearance. The species shows a great deal of variation, depending upon age, degree of calcification, and nature of the substratum.

SUMMARY

1. A total of 30 bryozoan species was reported from 37 species of marine algae.
2. Five bryozoan species were reported from three species of green algae, 26 bryozoan species from 11 species of brown algae, and 27 bryozoan species from 23 species of red algae.
3. *Phyllophora membranifolia* yielded the greatest number of bryozoan species (23), *Chondrus crispus* and *Laminaria Agardhii* each yielded 20, *Phyllophora Brodiaei*, 15, and *Cystoclonium purpureum cirrhosum* yielded 11 bryozoan species.
4. Each of the three commonest bryozoa, *Aetea sica*, *Boxerbankia gracilis* and *Crisia eburnea*, was found on 18 algal species.
5. Each of the two next commonest bryozoa, *Electra pilosa* and *Hippothoa hyalina*, occurred on 17 red and brown algal species.

6. *Bugula turrita*, the next most common form, was found on 16 algal species.
 7. *Crisia eburnea* and *Hippothoa hyalina* were common on over half the red algal species examined.

8. Some bryozoa seemed to grow most frequently and abundantly on certain algal species, namely:

- a. *Alcyonidium polyoum* on *Chondrus crispus*, *Phyllophora Brodiaei*, and *P. membranifolia*
- b. *Bowerbankia gracilis* on *Ascophyllum nodosum*, *Chondrus crispus*, *Fucus vesiculosus*, *F. vesiculosus spiralis*, *Phyllophora Brodiaei* and *P. membranifolia*
- c. *Crisia eburnea* on *Chondrus crispus*, *Phyllophora Brodiaei* and *P. membranifolia*
- d. *Electra pilosa* on *Laminaria Agardhii* and *Rhodymenia palmata*
- e. *Flustrella hispida* on *Ascophyllum nodosum*.

9. To the 84 known Woods Hole region bryozoan species can be added three more: *Aetea sica*, *Cellepora dichotoma* and *Scruparia ambigua*. It is quite possible that some of the previously reported *Aetea anguina* and *Cellepora americana* material may have included *Aetea sica* and *Cellepora dichotoma*, respectively.

10. Algal collections from New Rochelle, N. Y., yielded some of the same bryozoan species as are found in Woods Hole, namely: *Alcyonidium polyoum*, *Bowerbankia imbricata*, *Cryptosula pallasiana*, *Electra hastingsae*, *Membranipora lacroixii* (?), and *Pedicellina cernua*.

11. Algal collections from Rye, N. H., yielded some of the same bryozoa as are found at Woods Hole, namely: *Callopora aurita*, *Cribrilina annulata*, *Cribrilina punctata*, *Crisia eburnea*, *Electra pilosa*, *Hippothoa hyalina* and also a form, *Lichenopora hispida*, which did not occur at Woods Hole.

12. Twenty-six of the thirty bryozoan species were carefully illustrated.

13. Tentacle number counts were made for 15 species.

14. Three bryozoan species were collected, observed, or known to be in the larva-producing stage on algae in late June; seven species in July; ten in August; two in September. These were chance observations and the number of species would have been greater if more exhaustive collections over a greater number of months could have been made.

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STRATIFICATION AND DEFORMATION OF ARBACIA PUNCTULATA EGGS CENTRIFUGED IN CAFFEINE SOLUTIONS

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INTRODUCTION

During an investigation of the effect of methylated purines upon cellular behavior, the following problems arose: Does contact with the trimethylated purine, caffeine, which is known to influence cellular metabolism, affect the viscosity of the cytoplasm of the cell; and is there any evidence that this alkaloid influences the forces at the cell surfaces? Heilbrunn's informative studies (1926, 1928, 1943) on viscosity and surface forces, with reference to numerous chemical substances, did not deal adequately either with alkaloids as a group, or with caffeine in particular. Centrifugation followed by a study of the degree of granule stratification within the cytoplasm, together with changes in form of the cell itself, offered a satisfactory method of approach to these questions. From the experiments described below, it will be seen that caffeine does not alter the viscosity of the unfertilized egg but acts upon the membrane and cortical tension forces, thereby influencing cleavage in the fertilized egg. In the higher concentrations, even sperm entry is prevented by caffeine.

The temperature factor is not significant in the work reported here, since caffeinized eggs were centrifuged at the same time and temperature as control eggs from the same female. In this way, relative viscosity effects could be observed.

METHODS AND MATERIALS

The author (1945, 1946a, b, 1946) has demonstrated by studies on O_2 consumption and comparative sensitivity of developmental stages that caffeine retards cleavage in *Arbacia*. In the present study, the unfertilized and fertilized eggs [unfertilized in sea water (SW), unfertilized in 0.10 per cent caffeine-in-sea-water (CSW); fertilized, i.e. normal egg ($N \text{ } \text{fem}$) $\times$ normal sperm ($N \text{ } \text{masc}$), in sea water, and fertilized $N \text{ } \text{fem} \times N \text{ } \text{masc}$ in 0.10 per cent caffeine-in-sea-water] were centrifuged at $10,000 \times g$ for five, seven, and twelve minutes, and also at $3000 \times g$, 40 minutes after fertilization or at the equivalent time interval after shedding in the case of unfertilized eggs. This 40 minute period was chosen because that is when the viscosity of the protoplasm approaches the increased state typical at the time of cleavage. Comparable series employing other concentrations were also run.

Experiments were conducted at the temperature of running sea water. The appearance of uncentrifuged and centrifuged caffeinized eggs was compared with photomicrographs and descriptions by E. B. Harvey (1940). Differences in the degree of stratification (compactness) of the pigment granules and vacuoles and the height of the hyaloplasm zone after centrifugation were noted as evidence of

relative viscosities. To avoid error due to a time variable caused by the return of granules by Brownian movement, all photomicrographic records were made ten minutes after centrifugation.

RESULTS AND DISCUSSION

Cleavage abnormalities in eggs centrifuged in CSW were no greater than those observed in the same concentration of CSW without centrifugation. There was no evidence that caffeine induced any primary change in viscosity which would prevent cleavage. Clearly defined effects were reproducible and similar in both unfertilized and fertilized eggs, but those in the former were more convincing because the normal viscosity changes during mitosis made it impossible to assume that controls and experimentals would be in exactly the same state.

Bank (1932), using *Arbacia punctulata*, reported stratification within the unfertilized eggs without centrifugation if they were held in 1 per cent caffeine for 48 hours. This is not surprising since caffeine was shown by the author (1945, 1948) to retard the O_2 uptake of the *Arbacia* cell. The facility with which the egg contents stratify due to such a factor as O_2 uptake cannot be determined by centrifugation. In the experiments described in this paper, an indication of a surface effect was the fact that *Arbacia* eggs cannot be fertilized when immersed in 1 per cent CSW. Both the eggs and sperm, however, survive for a considerable period in 1 per cent CSW, and the eggs can be fertilized and undergo partial development if transferred to sea water. Therefore, this concentration of caffeine does not destroy the internal physiological potentialities of these gametes with respect to fertilization. Over as long a period as 48 hours, the physical effect noted by Bank can be understood on the basis of the biochemical inhibition of cellular respiration, and/or as a surface effect, without assuming a primary viscosity change due directly to caffeine.

Among the results observed in the present series, the delay of deformation, reduction in actual fragmentation, and the sharper margins of the layers (apparent under the earlier conditions of the experiments) were the most readily distinguishable and clearly associated phenomena. De Vries (1947), in his studies on viscosity and tension at the surface in eggs of the fresh water snail, *Limnaea stagnalis* L., based the interpretation of his results primarily on the occurrence of vacuoles and granules in the hyaloplasm zone, as well as on the height of this zone. He pointed out that the height of the hyaloplasm zone depends on both viscosity and the degree of stretching, i.e. tension at the surface. Therefore, it occurred to the present writer that the closer packing of the pigment granules might be attributable to the fact that the pigment in the spherical cell had a shorter distance to fall than the pigment in the uncaffeinated normal cell, which is always elongated by centrifugal force to the degree applied in the experiments up to this time. It seemed desirable to eliminate the effect of the stretching factor and resulting deformation in order to clarify the significance of the degree of stratification in interpreting viscosity changes. Accordingly, unfertilized and fertilized eggs, both control and experimental, were centrifuged at only $3000 \times g$ for intervals varying from one-half to three minutes. These shorter centrifugations at $3000 \times g$ did not change the shape of the cells in either the control or the

caffeinated eggs, but did allow stratification. Therefore, comparisons of stratification could be made without the deformation factor.

In the absence of internal viscosity changes, the increased force required to break caffeinated eggs indicates a surface effect. Harvey (1931) estimated that the centrifugal force necessary to pull the *Arbacia* egg into two halves indicates that tension at the surface for a 25 per cent increase in area is less than 0.2 dyne per cm. with considerable variation in eggs. At $10,000 \times g$ for 12 minutes, a count of five fields of each of the experimental caffeine series showed the percentage of breaking in the unfertilized eggs to be as follows: Controls in SW were 100 per cent broken; eggs in 0.02 per cent CSW, 12 per cent broken; in 0.10 per cent CSW, 0.50 per cent were broken; and in the 2.0 per cent CSW, only 0.08 per cent were broken although slight elongation did occur.

The apparent absence of any significant osmotic change (Cheney, 1948) as well as of demonstrable viscosity changes in the internal protoplasm, together with the delay in deformation reported here, would indicate that caffeine may initiate a change in the surface of the cell. Such an effect might involve both the membrane and the cortical protoplasm, which Harvey and Shapiro (1941) demonstrated to possess a considerably higher viscosity than the interior protoplasm in the eggs of *Arbacia punctulata* and *Asterias forbesii*.

SUMMARY *

1. Caffeine does not change the existing viscosity state of the egg.
2. Egg fragmentation, under centrifugation, decreases with increased caffeine concentration.
3. The "apparent" effect of greater stratification of the granules in *Arbacia* eggs centrifuged in caffeine does not occur if the centrifugal force to which the eggs are subjected is sufficient to produce sedimentation but insufficient to cause deformation.
4. Evidence indicates that the delay of deformation in the caffeinated eggs, centrifuged at $10,000 \times g$ or less, may be due to the action of caffeine (trimethylated purine) upon the total tension forces at the surface areas of both unfertilized and fertilized *Arbacia* eggs.

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A MUCIN CLOT REACTION WITH SEA-URCHIN FERTILIZIN

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INTRODUCTION

Recent work on the fertilizins (the sperm-agglutinating constituents of egg water) of the eggs of sea-urchins and other animals has shown them to be of the nature of mucoproteins. Tyler and Fox (1939, 1940) showed that the fertilizins of *Strongylocentrotus* and of *Megathura* possess protein characteristics, but are of low nitrogen content. Similar evidence has been obtained with *Arbacia* fertilizin by Kuhn and Wallenfels (1940) and with *Psannechinus* fertilizin by Runnström, Tiselius, and Vasseur (1942). The latter workers also obtained a positive carbohydrate test. Tyler (1948) reported the presence of reducing sugars to the extent of about 15 per cent in hydrolyzed, purified preparations of *Strongylocentrotus* fertilizin and identified galactose as one of the constituents. As will be shown later in this paper, the present author has found hexosamine to be present in amounts equivalent to about 2 per cent of the original material. According to Runnström, Tiselius, and Vasseur (1942) and Tyler (1946) the sea-urchin fertilizins are of pronounced acidic character.

Acidic mucopolysaccharides are known (cf. Meyer, 1945, and Stacey, 1946) to co-precipitate with proteins upon acidification of the native fluid or of the neutral extracts in the form of "mucin clots," stringy or granular precipitates, depending upon the conditions of precipitation. This reaction is given, for example, by hyaluronic acid (Meyer and Palmer, 1936) and has been used in the assay of the enzyme hyaluronidase (McClean, 1943). It was of interest to determine whether or not fertilizin preparations would give the mucin clot reaction. As the work reported here shows, fertilizin preparations do give such a mucin clot reaction. A titration method, based upon this, was developed for these preparations, and comparisons made with their sperm-agglutinating activity in untreated condition, after dialysis, and after exposure to heat and to ultra-violet irradiation.

MATERIALS AND METHODS

The sea-urchins *Lytechinus pictus*, *Strongylocentrotus purpuratus* and *S. franciscanus* were used in these experiments. Most of the work was done with *S. purpuratus*.

Two kinds of fertilizin preparations were employed. One, which will be termed "crude fertilizin," was prepared by acidifying a 20 per cent suspension of washed eggs to pH 3-3.5, removing the supernatant fluid after five or ten minutes and readjusting the pH of this solution to 7-7.5. The other, which will be termed "purified fertilizin," was further subjected to alkali precipitation, dialysis against 3.3 per cent acid saline (pH 3.5-4) and alcohol precipitation according to the method described by Tyler (1948). Material prepared in this manner has been

found to be electrophoretically homogeneous (Tyler, unpub.). For the various tests the solutions were made up in 3.3 per cent NaCl at a pH of about 7.

Sperm-agglutinating titer of the fertilizin preparations was determined using the drop method of preparing two-fold serial dilutions with sea water in Syracuse watch glasses. To two drops of each dilution of fertilizin solution one drop of a uniform sperm suspension, usually 1 per cent (calculated as 1 cc. dry sperm per 100 cc. sea water suspension), was added. The highest dilution in which agglutination is observable under the microscope gives the titer of the preparation.

Tests for univalent fertilizin were made according to the method described by Tyler (1941) and Metz (1942). Essentially, this method consists of first treating sperm with the solution containing univalent fertilizin, which does not agglutinate the sperm, and then adding an equal volume of strong normal fertilizin solution to the suspension of sperm. Failure of the sperm to be agglutinated by the normal fertilizin presumably indicates that the combining groups on the sperm surface have been occupied by univalent fertilizin groups and are no longer available to unite with the normal fertilizin. Univalent fertilizin was obtained by heating and by irradiation with ultra-violet light of normal fertilizin preparations (cf. Tyler, 1941, and Metz, 1942).

Bovine serum albumin prepared by the Armour laboratories was used in 1 per cent solution for the co-precipitation tests.

Hyaluronic acid was obtained from human umbilical cords according to the method described by McClean (1943), whereby the distilled water extract of acetone-dried, ground cords, extracted with 90 per cent acetic acid according to the method of Meyer and Palmer (1936), was precipitated with 1.25 volumes of cold, potassium acetate-saturated 95 per cent alcohol. The precipitate was washed with alcohol, acetone, and ether and dried over P_2O_5 . The dry product was dissolved in distilled water as required; a solution of 0.1–0.2 per cent of the dry material was clear, viscous and did not form a precipitate upon the addition of acetic acid, but co-precipitated with serum albumin in the presence of acetic acid, forming a stringy clot. In higher dilutions the mixture of serum albumin, acetic acid and hyaluronic acid solution resulted in the formation of a fine precipitate or turbidity. The highest dilution in two-fold serial dilutions in which turbidity was perceptible by visual inspection was taken as the titer of the hyaluronic acid solution.¹

EXPERIMENTS AND OBSERVATIONS

Co-precipitation of fertilizin with serum albumin in acid solution

A viscous solution of crude fertilizin of *Lytechinus pictus* was prepared as described above and combined with a 1 per cent solution of bovine serum albumin in 0.9 per cent NaCl and 2 N acetic acid according to the method described by McClean (1943) in the mucin clot test. With this solution a very large clot was formed similar in character and appearance to the clot formed by hyaluronic acid. Purified fertilizin preparations of *Strongylocentrotus purpuratus*, *S. franciscanus*, and *L. pictus* were tested in the same manner and in each case a clot or precipi-

¹ A quantity of pure potassium hyaluronate was later supplied to me by the Schering Corporation, through the courtesy of Dr. W. Alan Wright and Dr. Erwin Schwenk.

tate formed, depending upon the concentration of the solution. In Figure 1 a series of photographs of the mucin clot reaction of *S. purpuratus* fertilizin is shown, with the reaction given by hyaluronic acid for comparison.

By taking advantage of the biological activity of fertilizin, i.e., its sperm-agglutinating activity, a simple test was performed which provided definitive evidence that it is the fertilizin which is co-precipitated with serum albumin and not some hitherto undetected component of the material.

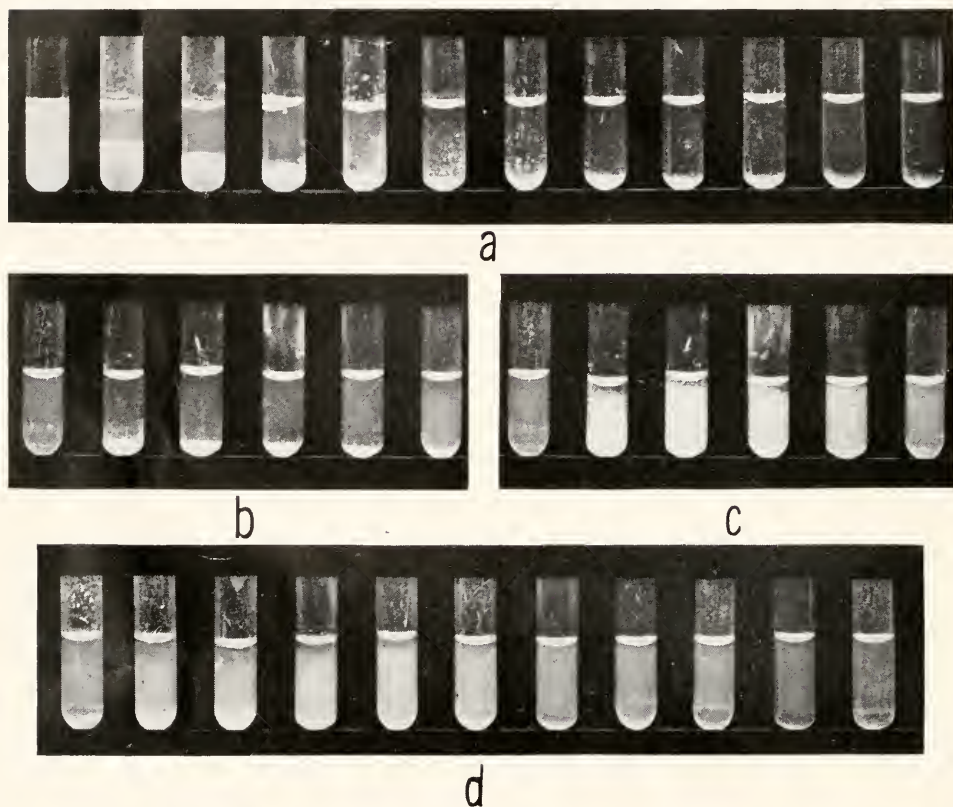


FIGURE 1. Mucin clot reactions of *Strongylocentrotus purpuratus* fertilizin and of hyaluronic acid in two-fold dilutions. a. Purified *S. purpuratus* fertilizin. The first tube contains fertilizin solution and serum albumin, but distilled water instead of acetic acid. The opacity of the first tube is due to the opalescence of the mixture. b. Crude *S. purpuratus* fertilizin. The first tube contains fertilizin solution and acetic acid but 0.9 per cent saline instead of serum albumin. c. The same as b with the tubes shaken prior to being photographed. d. Hyaluronic acid.

A solution of purified fertilizin in 3.3 per cent saline was mixed with serum albumin and the pH brought to about 3.5 with 2 N acetic acid. The resulting precipitate was thrown down by centrifugation, the supernatant withdrawn and its pH adjusted to 7. As shown in Table I, the supernatant exhibited no sperm-agglutinating activity.

TABLE I

Sperm-agglutinating activity of the supernatant and of the precipitate recovered separately after the addition of bovine serum albumin to an acidified solution of purified fertilizin, and results of control tests

Reaction mixture		Per cent of sperm-agglutinating activity*
0.5 ml. fertilizin in 3.3% saline	0.05 ml. distilled H ₂ O 0.2 ml. 0.9% saline	100
	0.05 ml. 2 N acetic acid 0.2 ml. 1% bovine serum albumin in 0.9% saline	Supernatant: 0 Dissolved precipitate: 100
	0.05 ml. distilled H ₂ O 0.2 ml. 1% bovine serum albumin in 0.9% saline	100
	0.05 ml. 2 N acetic acid 0.2 ml. 0.9% saline	100
	0.05 ml. distilled H ₂ O 0.2 ml. 0.9% saline, acidified and neutralized	100
	0.05 ml. 2 N acetic acid 0.2 ml. 1% bovine serum albumin in 0.9% saline	0

* Sperm-agglutinating activity of fertilizin, distilled water and 0.9% saline mixture taken as 100%.

It had previously been found that the precipitate formed by the addition of serum albumin to a fertilizin solution acidified to a pH of about 3.5 dissolves completely at a pH of 5.6 or higher. After centrifugation and withdrawal of the supernatant of the material being tested, the precipitate was resuspended in 3.3 per cent NaCl solution, its pH brought to about 7, and the volume made equal to that of the original mixture. Upon testing this solution it was found, as is shown in the table, that the sperm-agglutinating activity was equal to that of the original fertilizin solution, showing that the activity was recovered quantitatively from the precipitate.

A number of control tests were made, the results of which are summarized in Table I. These showed that (1) co-precipitation of fertilizin and serum albumin does not occur in the absence of acid; (2) it does not occur in acidified solution in the absence of added protein; (3) the presence of albumin does not affect the sperm-agglutinating activity of the fertilizin; (4) the sperm-agglutinating activity is not affected by acidification and subsequent neutralization of the solution, and (5) sperm agglutination does not occur in saline solution in the absence of fertilizin, nor is a precipitate formed when albumin and acid are added to saline.

Quantitative recovery of the sperm-agglutinating activity from co-precipitated fertilizin and serum albumin, which is achieved by the simple expedient of raising the pH to 5.6 or higher, shows that the specific combining groups of the fertilizin are not irreversibly altered by its reaction with the albumin. The significance of this phenomenon cannot be evaluated at the present time, however, since essentially nothing is as yet known of the structure of the fertilizin molecule.

Titration by the mucin clot method

The method employed for titration was as follows: Two-fold serial dilutions of fertilizin in 3.3 per cent NaCl at about pH 7 are made in 10×75 mm. test tubes in 0.5 ml. quantities. To each tube 0.05 ml. of 2 N acetic acid is added and the contents mixed. The tubes are inclined and 0.2 ml. of 1 per cent bovine serum albumin in 0.9 per cent saline is slowly pipetted down the side of each tube; after this the tubes are carefully returned to a vertical position and in most cases a ring of precipitate forms immediately at the zone of contact between the albumin solution and the acidified fertilizin solution. The rings are easily detected at high dilutions in which a diffuse turbidity is difficult to detect and score visually. The reactions are read at once in good natural light, since the rings tend to disperse rapidly as the albumin diffuses through the mixture. The highest dilution at which a ring is observed is taken as the mucin clot titer of the preparation.

Effect of pH, albumin concentration and salt concentration

Numerous titrations with the same stock samples of purified fertilizin using the method described above have given consistently identical mucin clot titers in the course of routine testing. It was thought advisable, however, to carry out some controlled tests to determine in a more definitive manner the amount of variability in titer to be expected within a rather limited range of pH, albumin concentration, and salt concentration.

A sample of a purified fertilizin solution from eggs of *S. purpuratus* was centrifuged at 3000 r.p.m. for five minutes. A slight sediment was thrown down, the clarified supernatant was drawn off, and the pH adjusted to 7.0 with the glass electrode. To assure maximum uniformity of different samples of the supernatant, it was thoroughly mixed before removing an aliquot. Two-fold dilutions were made in 0.5 ml. quantities with 3.3 per cent NaCl solution. Serum albumin was made up in 1 per cent solutions, and the same stock solution of 2 N acetic acid was used throughout. Various amounts of the latter two solutions were added to different sets of tubes of the fertilizin dilutions. The total albumin and salt concentration in the different sets was thus altered, as noted below. The pH was measured with a Beckman glass electrode pH meter. After each titration the pH of the final mixtures was measured in the first tube, in the tube giving the end-point, and in an intermediate tube. The maximum difference in pH observed within any set was 0.26 unit. The results obtained in a series of nine titrations are given in Table II. Each of the pH values listed represents the mean of three determinations in each set. The total salt concentration is expressed as per cent NaCl. The mucin clot titers (last column of table) are the end points of visible precipitation as determined by the ring method described above.

As the data in the table show, no marked difference in mucin clot titer occurs, for the most part, as a result of the differences in pH, total salt and albumin concentrations employed. In titration 5, the observable end-point would probably have been at least one dilution higher had rings formed. The failure of rings to form is attributable to the relatively large quantity of serum albumin solution used; as noted above, in high dilutions a diffuse turbidity such as was produced in this case is more difficult to detect visually than a ring at the same dilution. It has

been found that 0.5 ml. of albumin solution is about the largest quantity practicable for obtaining consistent ring formation. Since there is always a tendency to pipette too rapidly when a large number of tests is being performed, it has proven more convenient to use 0.2 ml. of albumin solution.

The evidence presented in Table II indicates that pH is not a critical factor with respect to observed titer within the range tested. The albumin concentration is not critical, nor is the total salt concentration of the system in the range from 2.0 per cent to 3.1 per cent. Below 2.0 per cent the salt concentration may be a more important factor, as shown by the higher titer obtained in titration 6. Although the total salt concentration in titration 5 is the same as that in number 6 (1.6 per cent), the two sets are not comparable for the reason mentioned above.

TABLE II

Mean pH, total albumin concentration, total salt concentration and mucin clot titers in nine titrations using uniform samples of a homogeneous purified fertilizin preparation of S. purpuratus

Titration no.	Ml. 2 N acetic acid	Ml. 1% albumin solution	NaCl concentration of albumin solution, per cent	Mean pH	Total albumin concentration, per cent	Total NaCl concentration, per cent	Mucin clot titer
1	0.05	0.1	0.9	3.09	0.15	2.7	64
2	0.05	0.2	0.9	3.30	0.27	2.4	64
3	0.05	0.3	0.9	3.33	0.35	2.3	64
4	0.05	0.5	0.9	3.54	0.47	2.0	64
5	0.05	1.0	0.9	3.74	0.65	1.6	32*
6	0.05	0.5	0.0	3.58	0.47	1.6	128
7	0.05	0.5	3.3	3.58	0.47	3.1	64
8	0.01	0.2	0.9	3.88	0.28	2.6	64
9	0.02	0.2	0.9	3.78	0.28	2.5	64

* Scored as turbidity, not as ring.

In the next section it will be shown that the mucin clot and sperm-agglutinating titers of a fertilizin solution are both reduced after dialysis of the preparation against distilled water with consequent removal of salt (NaCl). The data to be presented indicate that the physical state of the fertilizin is reversibly modified in the absence of electrolytes, at least under certain conditions of temperature. It seems likely, therefore, that there may exist some optimum concentration of electrolytes between zero concentration and that represented by 2.0 per cent NaCl (equivalent to an ionic strength of 0.34) at which co-precipitation of fertilizin and serum albumin attains a maximum. Further analysis of the effect of salt concentration on the co-precipitation of fertilizin and serum albumin is under way and will be reported in a later communication.

In the system as employed in the routine titration procedure in the present investigation, however, the mean pH, total albumin and total salt concentration fall well within the range for each of these factors in which identical titers are obtained, using uniform samples of a homogeneous fertilizin preparation.

Mucin clot and sperm-agglutinating titrations of fertilizin in salt-free solution

A sample (I) of a stock preparation of purified fertilizin in 3.3 per cent saline was made salt-free by dialyzing against distilled water at 1° C. until it no longer formed a precipitate with AgNO_3 . It was found that a white, flocculent material was present in the dialyzed solution, whereas no such flocculence was evident in the control in 3.3 per cent NaCl solution which was kept at 1° for the same period. A considerable reduction in both mucin clot and sperm-agglutinating titer of the salt-free sample as compared with the control was observed. In the mucin clot titration, the character of the precipitate formed in the salt-free preparation upon the addition of serum albumin in the presence of acid differed from that in the control in being of larger particle size and somewhat stringy. Difficulty was encountered in making sperm-agglutinating titrations (where the salt content of the preparation was adjusted just prior to titration to 3.3 per cent NaCl by adding an equal volume either of 6.6 per cent NaCl or of double sea water to the salt-free solution) in cases where suspended material was present. The spermatozoa clumped about the particulate matter, and it was impossible to score the dilutions satisfactorily. Where tests were made with samples of the dialyzed solution in which the amount of suspended material was visibly less than was originally present, both the mucin clot precipitation and sperm agglutination occurred in the manner characteristic of the control solutions; in these cases the two titers were also somewhat higher, although they did not necessarily equal the values obtained for the control solution.

A second sample (II) of the same stock preparation of purified fertilizin was dialyzed against distilled water until salt-free. This time the first five changes of the water used for dialysis, totaling 3 liters, were saved, combined, and lyophilized to dryness. The residue was taken up in about 10 cc. of distilled water and the resulting solution was approximately isotonic with sea water, as shown by the fact that sperm of *S. purpuratus* remained active when placed in it. This "dialysate-concentrate" was 300 times more concentrated than the original dialysate, but proved to be negative for both mucin clot formation and sperm-agglutinating activity. Sample II behaved in all respects like sample I. The results of tests with the two samples are summarized in Table III.

The evidence obtained from the present experiments indicates that at temperatures near the freezing point (1° C.) the physical state of fertilizin can be reversibly modified by the removal of electrolytes. Macroscopic aggregates may appear in a fertilizin preparation under these conditions, and there is a correlated decrease in the mucin clot and sperm-agglutinating titers. Under the influence of added salt and elevated temperature (up to 21.5° C.), either separately or combined, there occurs a correlated decrease in the amount of visible macroscopic material and increase in mucin clot and sperm-agglutinating titers to values approaching those obtained with control solutions. The negative results of tests with the "dialysate-concentrate" show that there was no actual loss of fertilizin during dialysis.

Analysis of a purified fertilizin preparation

The co-precipitation of fertilizin with protein in acidic solution in a manner analogous to the behavior of acid mucopolysaccharides suggests affinity of fertilizin with this class of substances. It has recently been claimed, moreover, that a preparation

TABLE III

Results of titrations of salt-free fertilizin preparations of S. purpuratus after various treatments

Test no.	Fertilizin preparation	Treatment after removal from dialysis bath at 1° C.	Sperm-agglutinating titration		Mucin clot titration	
			pH	Titer	pH	Titer
1	I	None				
2	I	Salt content adjusted to 3.3% NaCl. pH adjusted	7.2	64	5.2	8
3	I	Salt content adjusted to 3.3% NaCl			5.2	16
4	I	2.5 hrs. at 21.5° C.			5.2	16
5	I	2.5 hrs. at 21.5° C. pH adjusted			7.2	16
6	I	Dialyzed vs. 3.3% NaCl at 1° C. 2 hrs. at 21.5° C. pH adjusted	7.0	64	7.0	16
7	I	Dialyzed vs. 3.3% NaCl at 1° C. 24 hrs. at 8° C. pH adjusted	7.45	1024	7.45	32
8	I	Control. Not dialyzed vs. distilled water. pH adjusted	7.4	512	7.4	64
9	II	1 hr. at 21.5° C. pH adjusted			6.9	32
10	II	Salt content adjusted to 3.3% NaCl. 3 hrs. at 21.5° C. pH adjusted	6.9	512		
11	II	Salt content adjusted to 3.3% NaCl. 1 hr. at 21.5° C. pH adjusted			6.9	64
12	II	Control. Not dialyzed against distilled water. pH adjusted	7.3	1024	7.3	128

from bull testes, presumably containing the enzyme hyaluronidase, is capable of causing the jelly of intact sea-urchin eggs to swell (Ruffo and Monroy, 1946; Monroy and Ruffo, 1947). It was of interest, then, to attempt to determine the extent to which fertilizin may be chemically similar to hyaluronic acid. The few available data, which have been reviewed briefly in the introductory section, indicate that fertilizin is by no means identical with hyaluronic acid. The present investigation has shown that fertilizin differs from hyaluronic acid to a marked degree with respect to the two chief constituents of the latter substance, hexosamine and glucuronic acid.

A homogeneous sample of a purified fertilizin preparation in 3.3 per cent NaCl was prepared as described above. In the present instance the supernatant fluid was filtered through hardened filter paper. The pH of the filtrate was adjusted to 7 and

TABLE IV

Results of chemical analysis of a purified fertilizin preparation of S. purpuratus

Filtrate					Hydrolysate	
Sperm-agglutinating titer	Mucin clot titer	Dry weight, mg./ml.	Total nitrogen, per cent	Glucuronic acid	Hexosamine, per cent	α -amino acids
2048	512	8.95	4.1	none	1.6	positive Ninhydrin

mucin clot and sperm-agglutinating titers were obtained. Aliquots of the filtrate were taken for the various analyses, which included determinations of dry weight, total nitrogen, hexosamine, α -amino acids and glucuronic acid. The results of the analyses are presented in Table IV. Dry weight per ml. was calculated from the weight of material precipitated from an aliquot of the filtrate with 1.25 volumes of cold 95 per cent alcohol. According to Tyler (1948), precipitation of fertilizin is complete under these conditions. The precipitate was washed with alcohol and dried in an oven at 55° C. to constant weight. Total nitrogen was determined for duplicate samples of the dried precipitate by the micro-Kjeldahl method. Another portion of the dried material was hydrolyzed by boiling in a sealed tube with 4N HCl for eight hours. Hexosamine was determined in an aliquot of the hydrolysate by the method of Palmer, Smyth and Meyer (1937). Another portion of the hydrolysate was treated with Ninhydrin reagent for the determination of α -amino acids. For the determination of glucuronic acid the colorimetric method recently described by Dische (1947b) was employed, using a sample of the original filtrate,

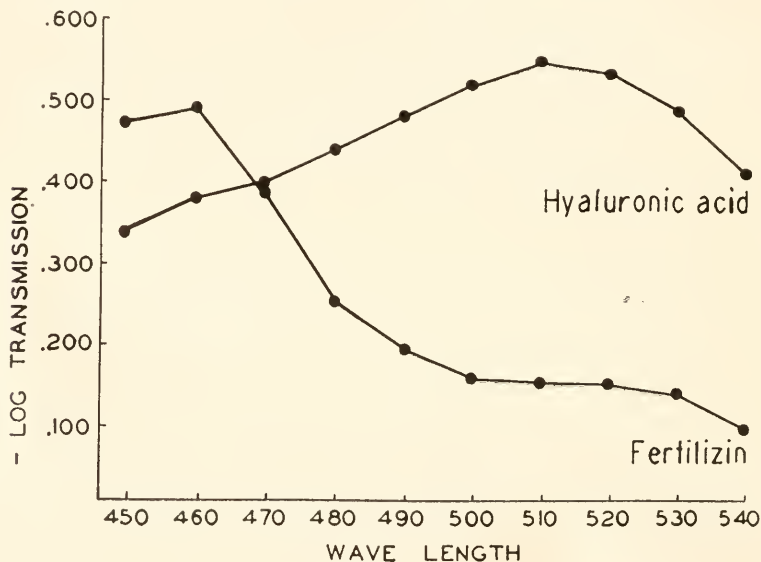


FIGURE 2. Absorption curves of reaction mixtures of mannose-thioglycolic acid-fertilizin and mannose-thioglycolic acid-hyaluronic acid. Wave lengths in $m\mu$. Fertilizin $\Delta E_{(510-480)} = -0.097$; hyaluronic acid $\Delta E_{(510-480)} = +0.115$.

and for comparative purposes, a solution of pure potassium hyaluronate (Schering) was tested at the same time by the same method. According to Dische, the reaction of carbohydrates with -SH compounds in H_2SO_4 , which differentiates between various classes of carbohydrates and individual hexoses and hexuronic acids, is highly characteristic for glucuronic acid when mannose is employed. This reaction is the basis of the test. The reaction mixture with glucuronic acid gives a typical absorption curve in the range 450–540 $m\mu$, and it was found by Dische that the curve for hyaluronic acid is almost identical with that of glucuronic acid. In practice, accord-

ing to Dische, it is only necessary to measure the intensity of the mannose reaction at 510 and 480 $m\mu$ and subtract the second value from the first. This difference is positive for glucuronic acid and polyglucuronides, and negative for the other hexuronic acids. Figure 2 shows the absorption curves for fertilizin and for hyaluronic acid. The difference between the intensity of the mannose reaction with fertilizin at 510 and that at 480 $m\mu$ is negative, and hence it may be concluded that fertilizin does not contain glucuronic acid. This result is in agreement with previous results obtained by Tyler (unpub.) using an earlier method of Dische's (1947a). As shown in Table IV, fertilizin does contain hexosamine, but in small amount, which is in agreement with earlier results obtained by the present author using Kunitz's (1939) method. The Ninhydrin reaction was very weak but probably positive. Total nitrogen (4.1 per cent) of this material is somewhat lower than has been reported previously for *S. purpuratus* fertilizin by Tyler and Fox (1940), who found an average total nitrogen content of 5.2 per cent with crude preparations.

These data show that fertilizin differs markedly from hyaluronic acid in its chemical constitution. It is obvious, therefore, that the ability to give the mucin clot reaction does not by any means indicate close similarity between fertilizin and hyaluronic acid, even though it may be evidence that the former is related to the group of acidic mucopolysaccharides.

At the suggestion of Dr. Albert Tyler, the mucin clot titration procedure was used in conjunction with sperm-agglutinating titrations to investigate the effects of various kinds of treatment on fertilizin. In the following sections the results of parallel titrations of preparations subjected to heat and to ultra-violet irradiation are presented.

Parallel titrations with heat-treated fertilizin solutions

According to Tyler and Fox (1940), the sperm-agglutinating activity of *Strongylocentrotus purpuratus* fertilizin is rapidly destroyed at 100° C. The rate of inactivation, according to these authors, is a function of the pH, the fertilizin being most stable in the range from 4 to 7. Their data show that at pH 7.3 the agglutinin is 95–100 per cent inactivated in 20–30 minutes at 100°. In the present experiments in which *S. purpuratus* purified fertilizin solutions were used, the preparations have proven to be considerably more heat-stable than the material used by Tyler and Fox. Since the solutions employed by Tyler and Fox corresponded to crude fertilizin as defined in this paper, it may well be that the relatively purer condition of the fertilizin in the present preparations accounts for its greater stability.

Initial loss of agglutinating activity does not appear to involve complete destruction of fertilizin. At first the agglutinating fertilizin is converted into a "univalent," non-agglutinating form (cf. Tyler, 1941, Metz, 1942). It was of interest, accordingly, to test samples of heat-treated fertilizin for their univalence (inhibition) titer as well as for their sperm-agglutinating and mucin clot titers. The method employed for detecting univalent fertilizin has been briefly described in an earlier section of this paper; determination of inhibition titer consists in determining the greatest dilution in which no agglutination occurs upon the addition to the test dilutions of equal amounts of normal (untreated) fertilizin solution (Metz, 1942).

In the first experiments 1.5 ml. samples of the stock purified fertilizin solutions were placed in 13 × 100 mm. test tubes and immersed in a boiling water bath. The

exposed portions of the tubes were cooled by means of a stream of air so that heating could be continued for long periods without appreciable loss of fluid. Since sperm are quickly inactivated in even slightly hypertonic medium, the fact that the sperm remained active in the solutions that had been heated was assumed to indicate that evaporation of water from the tubes during heating was insignificant. A thermometer placed in the water bath with the tubes showed that the temperature of the bath fluctuated between 96° and 98° C.

In later experiments a quantity of fertilizin solution large enough to permit the withdrawal of a number of 1.5 ml. samples was placed in a flask with a reflux condenser attached by means of a ground glass joint. The solution was refluxed and loss of water was thus kept to a minimum. The temperature of the boiling fluid in the flask could be assumed to be about 100° C. A considerable excess of solution was used so that its concentration would not be significantly affected by the slight amount of water that failed to run back down. Before withdrawing a sample, the neck of the flask and the lower part of the condenser were cooled with cold water from a wash bottle. In all of the experiments the pH of the fertilizin solutions was adjusted with the glass electrode just before heating was begun; a control sample was allowed to stand at room temperature throughout the total time of heating. As each sample was removed from the water bath or from the reflux flask,

TABLE V

Results of parallel titrations of heat-treated, purified fertilizin preparations of S. purpuratus

Fertilizin preparation	Initial pH	Sample	Temperature, degrees Cent.	Time in hours	Final pH	Titer		
						Sperm agglutination	Mucin clot	Inhibition
I*	7.1	a	96-98	0.5	—**	256	64	—**
		b	96-98	1.0	—	64	64	—
		Control	room	1.0	—	512	64	—
II*	7.85	a	96-98	4.5	—	16	64	—
		Control	room	4.5	—	256	64	—
III*	7.4	a	96-98	3.5	—	128	256	0
		b	96-98	5.5	—	64	64	32
		Control	room	5.5	—	1024	256	—
IV***	7.5	a	100	2.0	7.5	4096	512	—
		b	100	3.0	7.2	4096	512	—
		c	100	5.0	7.1	2048	256	—
		d	100	6.0	7.1	2048	256	0
		e	100	7.5	7.1	1024— 2048****	256	0
		Control	room	7.5	6.8	4096	512	—

* Individual 1.5 ml. samples heated in water bath.

** Dashes indicate not tested. Zero inhibition titer indicates tested but inhibition not detected.

*** Samples (1.5 ml.) withdrawn from refluxed solution.

**** A trace reaction probably present in the higher dilution.

it was placed immediately in the freezer. In the later experiments the pH of each heated sample was recorded before it was frozen. The titrations were performed as soon thereafter as possible. The results of the experiments are presented in Table V.

As may be seen from the table, reduction of sperm-agglutinating titer by heating is not necessarily accompanied by parallel reduction in mucin clot titer. Thus, for example, samples IIa and IIIa, heated for 4.5 and 3.5 hours respectively, showed no significant reduction in mucin clot titer although the sperm-agglutinating titer of the former was reduced to about 6 per cent and that of the latter to about 12 per cent of the original values. Sample IIIb was heated for 5.5 hours with a reduction of sperm-agglutinating titer to approximately 6 per cent of its original value. In this case the mucin clot titer was reduced to 25 per cent of the original value. Samples IVa-IVe show a more nearly parallel reduction of sperm-agglutinating and mucin clot titers than any of the others. Preparation IV was refluxed. After boiling for 7.5 hours the sperm-agglutinating titer was reduced to 25-50 per cent of the original value and the mucin clot titer was reduced to about the same per cent of the original value. Sample IIIb was the only one which gave an inhibition (univalence) titer. In the samples which were tested for inhibition but in which none was detected (IIIa, IVd, IVe), it is probable that insufficient univalent fertilizin was present in the high dilutions to permit detection. The inhibition test is unambiguous only in dilutions containing sufficient univalent fertilizin to react with most of the added sperm. In the high dilutions enough sperm remain uncombined to be agglutinated upon the addition of normal fertilizin and thus obscure the slight amount of inhibition that may be present. In the present experiments, sample IIIb was the only one in which sufficient univalent fertilizin was produced in the lower dilutions to give clear-cut evidence of inhibition. Since the inhibition titer of IIIb was 32, while the mucin clot titer was 64, it appears that the mucin clot reaction of fertilizin does not depend upon maintenance of the multivalent condition. Stronger evidence to support this view was afforded by experiments in which fertilizin was irradiated with ultra-violet light.

Parallel titrations with ultra-violet irradiated purified fertilizin preparations

Metz (1942) showed that univalent fertilizin is produced by irradiation of normal (multivalent) fertilizin by ultra-violet rays. In the present experiments ultra-violet irradiation was carried out in an apparatus consisting of glass tubing, 150 × 35 mm., fitted on the mid-section of a 15 watt General Electric "Germicidal" lamp, the diameter of which is 25 mm. The major part of the output of this lamp is concentrated in the 2537 Å wave-length band. The space between the outer wall of the lamp and the inner wall of the tubing is the irradiation chamber. The chamber and lamp assembly is mounted on a motor-driven rocker. An opening in the top of the chamber, which can be closed with a rubber stopper, permits the introduction and withdrawal of fluid. The chamber is cooled by means of a small electric fan mounted on the rocker platform; when the fan is in operation the temperature of fluid inside the chamber does not rise above 35° C. during irradiation.

In the first experiment, a purified fertilizin preparation of *S. purpuratus*, the pH of which was first adjusted to 7, was irradiated for a total of 2.5 hours. It was found, as shown in Table VI, that the sperm-agglutinating titer was reduced to

TABLE VI
Results of ultra-violet irradiation of purified fertilizin

Fertilizin preparation	Sample no.	Initial pH	Time of irradiation in hours	Final pH	Sperm agglutination titer	Mucin clot titer	Inhibition titer
I	Control 1	7.0	—	—	256	64	—
		7.0	2½	—	4	32	—*
II	Control	7.67	—	6.60	1024	1024	—
	1	7.67	4½	5.52	0	1024	128
	2	7.67	6	5.49	0	1024	128
	3	7.67	7¾	5.49	0	512	4
	4	7.67	9	5.49	0	256	4

* Univalence present in this sample by inhibition test but titer not obtained.

about 2 per cent of the original value and the mucin clot titer was decreased to 50 per cent of the original. Tested by the inhibition method, the irradiated preparation was found to contain univalent fertilizin. The inhibition titer of this sample was not obtained. In a second experiment, a quantity of the fertilizin preparation which was found to be very heat-stable with respect to its sperm-agglutinating activity (preparation IV of the preceding section) was irradiated. The pH of the solution was first adjusted to 7.7. Small portions (1.5 ml.) were withdrawn at intervals up to nine hours; the first sample was removed after 4.3 hours of irradiation. A control sample was allowed to stand in natural light (filtered through window glass) at room temperature throughout the entire period of irradiation. Immediately upon the removal of each sample from the irradiation chamber, its pH was measured with the glass electrode, and then it was placed in the freezer. All of the samples, including the control, were stored in the freezer until the titrations could be performed. As shown in Table VI, all of the irradiated samples showed complete loss of agglutinating activity. Tested by the inhibition method, all of them were found to contain univalent fertilizin. The inhibition titers showed a progressive decrease as time of irradiation was increased. The mucin clot titers also showed a progressive decrease with increased time of irradiation. After nine hours the mucin clot titer was reduced to 25 per cent of its original value, and the inhibition titer was reduced to about 6 per cent of the value found after 4.3 hours of irradiation.

The results of these experiments demonstrate conclusively that the mucin clot reaction of fertilizin does not depend upon maintenance of the multivalent condition. They also show that ultra-violet irradiation is a more effective agent than heat in converting multivalent, purified fertilizin to the univalent condition. The progressive decrease in inhibition titer found in the second experiment indicates that degradation of the fertilizin by ultra-violet light proceeds beyond the stage in which it exhibits univalence.

DISCUSSION

In general it may be said that the mucin clot titer of untreated fertilizin preparations parallels their sperm-agglutinating activity. Sperm agglutination is usually detectable in higher dilutions than is the mucin clot reaction where the latter is observed by the ring method used in the present experiments.

Destruction of the sperm-agglutinating activity of fertilizin is not necessarily accompanied by a reduction of mucin clot titer. Conversely, however, it is clear that fertilizin which has been subjected to treatment that causes a reduction in mucin clot titer, for example heating or irradiation by ultra-violet light for extended periods, will invariably show at least a parallel decrease in sperm-agglutinating activity. It has been shown in the experiments with ultra-violet irradiation that the capacity of the fertilizin to agglutinate sperm may be completely destroyed with but little, if any, loss of its ability to give the mucin clot reaction. The evidence shows that when the agglutinating (multivalent) form is degraded to the non-agglutinating (univalent) form, the latter continues to co-precipitate with protein in the mucin clot reaction. If a preparation in which all of the fertilizin has been made univalent is subjected to continued irradiation by ultra-violet light, a progressive decrease in both mucin clot and inhibition titers occurs.

The phenomenon of sperm agglutination by fertilizin has been interpreted by Tyler (1941, 1942, 1947, 1948) as an antigen-antibody type of reaction in which complementary combining groups of a substance (antifertilizin) on the surface of the sperm cells unite in "lock and key" fashion with the combining groups of fertilizin. Where a number of such combining groups are available on the surface of the fertilizin molecule, agglutination occurs as the result of the building up of a lattice, as postulated for analogous immunological reactions by Heidelberger (1938) and Marrack (1938). The formation of univalent fertilizin is brought about by various agents—e.g. heat, ultra-violet light, x-rays—which, according to Tyler (1941), split the molecule into fragments, each of which contains a single combining group. These fragments are still of large size, since they are non-dialyzable (Tyler, 1941). They are also capable of co-precipitating with protein in the presence of acid, giving the mucin clot reaction.

The ability to give the mucin clot reaction is, at least in the case of hyaluronic acid, presumably a function of the degree of polymerization of the molecule (Meyer, 1947). Depolymerized molecules are incapable of giving the reaction. Although fertilizin has been shown to be very different from hyaluronic acid in its chemical composition, the fact that it co-precipitates with protein in acid solution in an analogous manner suggests that it may be similar in its physical structure. Thus, fertilizin may normally exist in a polymerized condition. Sperm-agglutinating activity may, then, accompany a range of polymer size, and the univalent condition may represent a state of polymerization with which but a single combining element is associated. Degradation of multivalent fertilizin to the univalent form would then entail a progressive splitting off of relatively stable univalent units. The evidence from the experiments with ultra-violet irradiation indicates that the univalent form is in fact the more stable, since complete conversion to univalence was observed after 4.3 hours of irradiation, whereas even after nine hours both the inhibition and mucin clot titers retained significant values.

SUMMARY

1. Preparations of fertilizin of three species of sea-urchin have been found to give a mucin clot reaction similar to that given by hyaluronic acid. Upon the addition of bovine serum albumin to an acidified solution of fertilizin, a precipitate forms which dissolves at a pH of 5.6 or higher. All of the sperm-agglutinating activity accom-

panies the precipitate and it is recovered quantitatively when the precipitate is dissolved.

2. A method for the determination of mucin clot titer of fertilizin is described.

3. At temperatures near the freezing point (1°C.) the physical state of fertilizin can be reversibly modified by the removal of electrolytes by dialysis. Macroscopic aggregates appear, accompanied by a parallel decrease in mucin clot and sperm-agglutinating titers. Disappearance of the aggregates is accompanied by an increase in both titers.

4. Chemical analysis of fertilizin shows that it contains no glucuronic acid, about 2 per cent hexosamine and amino acids. Fertilizin, therefore, differs greatly from hyaluronic acid, but its ability to give the mucin clot reaction suggests an affinity with the class of mucopolysaccharides.

5. In general, mucin clot titer parallels sperm-agglutinating titer of the same untreated fertilizin preparation, although sperm agglutination is detectable in higher dilutions than is the mucin clot reaction where the latter is observed by the ring method used in the present experiments.

6. Parallel mucin clot and sperm-agglutinating titrations were made with fertilizin preparations in untreated condition and after exposure to heat and to ultra-violet irradiation. The purified preparations used in these experiments proved to be exceptionally heat-stable; irradiation by ultra-violet light was found to be a more effective treatment in degrading the material.

7. Destruction of the sperm-agglutinating activity of fertilizin by heat and by ultra-violet irradiation does not necessarily cause a parallel decrease in mucin clot titer. The mucin clot reaction continues to be given by preparations in which all of the fertilizin has been converted from the normal, agglutinating condition to the non-agglutinating, "univalent" form. Continued irradiation of the univalent fertilizin is accompanied by a progressive decrease in both inhibition and mucin clot titer.

8. It is suggested that fertilizin may normally exist in a polymerized condition and that the non-agglutinating, "univalent" condition may represent a relatively more stable lower polymer of the native, agglutinating form.

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THE ANAPHASE MOVEMENT OF CHROMOSOMES IN THE SPERMATOCYTES OF THE GRASSHOPPER

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Among the many complex processes involved in the division of cells, the movement of the chromosomes at anaphase is most accessible to a causal analysis. The beautiful preciseness of the processes involved in the orderly separation of chromosomes has for a long time enticed biologists to search for their physico-chemical basis. However, before an analysis on this level is possible, it is necessary, first, to know what structural differentiations of the cell are involved, and secondly, to have detailed quantitative descriptions of the processes based on a study of living cells.

A previous analysis of chromosome movement in certain insects (Homoptera and Hemiptera) has shown that the structures involved in anaphase movement are the kinetochores on the chromosomes, the chromosomal fibers, which connect the kinetochores to the spindle, and the spindle body (Ris, 1943). The kinetochore determines the nature of the chromosomal fibers, which in the case of these insects are broad and sheet-like and attached to the entire length of the chromosome (cf. Hughes-Schrader and Ris, 1941). The movement of the chromosomes consists of two separate processes: first, the shortening of the chromosomal fibers, which moves the chromosomes to the poles of the spindle; and secondly, the elongation of the spindle body, which further separates the chromosomes. In the Homoptera and Hemiptera these two components of anaphase movement are separated in time, so that first the chromosomes move to the poles of the spindle and then, after a pause of a few minutes, the spindle body stretches and carries the chromosomes further apart.

In most animals and plants the chromosomal fibers are narrow bundles attached to a definite, restricted region of the chromosome. In this paper the spermatocyte divisions of the grasshopper were chosen in order to analyze chromosome movement in an organism with localized kinetochore.

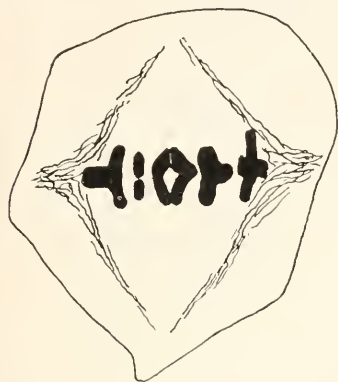
MATERIAL AND METHODS

The measurements recorded here were made on spermatocytes of *Chorthophaga viridifasciata*. A few measurements on *Dissosteira carolina*, *Melanoplus femurrubrum*, *Arphia xanthoptera* and *Hippiscus spec.* gave similar results.

The spermatocytes of the grasshopper are classical material in the study of living cells in division (Chambers, 1914, 1924; Lewis and Robertson, 1916; Belar, 1929; Baumgartner and Payne, 1931). The usual technique consisted in breaking the testis follicles and spreading the cells on a coverglass in Locke's or Ringer's solution. Baumgartner and Payne (1931) showed that the follicles can be left intact and the cells studied with high powers. They pulled the testis through an opening

¹ Part of the work for this paper was done in the Department of Biology, Johns Hopkins University.

of the body wall into a little pool of salt solution, but left it attached to the vasa efferentia. Since they had to remove the follicular membrane which contains the trachae, the testes may as well be completely removed from the animal. In the present work the testes were dissected out, the follicular membrane removed, and the follicles spread intact on a coverglass into a drop of Belar's solution (Belar, 1929). The coverslip was inverted over a depression slide and sealed with paraffin. Aseptic technique was not attempted since only preparations made on the same day were used for measurements. The temperature was kept constant at 30° C. with an electric stage warmer. A liquid filter of ferrous ammonium sulphate prevented heat from the lamp from reaching the object. The cells, thus, were disturbed as little as possible.



1



2

FIGURES 1 and 2. Camera lucida drawings of living spermatocytes of *Chorthophaga*. Side view of primary and secondary spermatocyte metaphases.

To measure the movement of chromosomes a metaphase in side view was selected, and as soon as the chromosomes began to separate, the distance between kinetochores was recorded at regular intervals with a camera lucida. In primary spermatocytes a bivalent with terminalized chiasmata near the spindle axis was chosen. In the secondary spermatocytes a chromosome in a median optical section of the spindle was selected. Though the spindle itself is hardly visible, it is clearly outlined by the chondriosomes (Figs. 1, 2). This makes it possible to measure the length and the equatorial diameter of the spindle during the entire anaphase. The various distances were then plotted against time, yielding a curve which describes the movement of the chromosomes and the changes in spindle length and diameter. All measurements were made with a 4 mm. Zeiss apochromat and 15 × ocular.

OBSERVATIONS

Anaphase movement in the first spermatocyte division

In Figure 3, three out of thirteen measured cells are presented.² The distance between separating kinetochores is plotted against time. The resulting curve con-

² The curves from different cells, even coming from different individuals, agree remarkably well, especially in the beginning of chromosome movement before spindle stretching sets in. The three cells shown indicate the degree of variation.

sists of an initial slow movement, then a straight portion of maximum velocity and a less regular part of gradually decreasing movement. Finally, before the cleavage furrow appears, the chromosomes move together again for a short distance, apparently due to the shrinkage of the spindle.

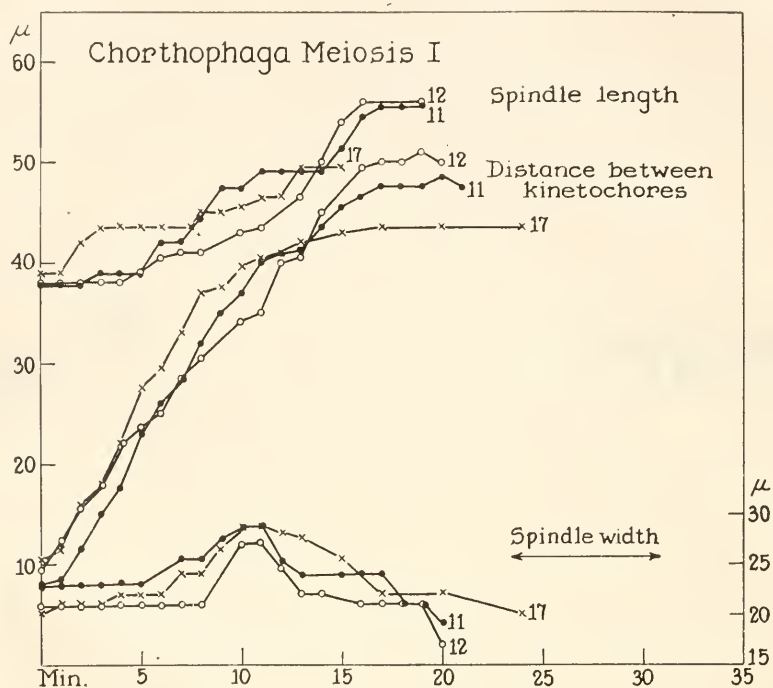


FIGURE 3. Chromosome movement and spindle behavior in the first meiotic division of *Chorthophaga viridifasciata*. See text.

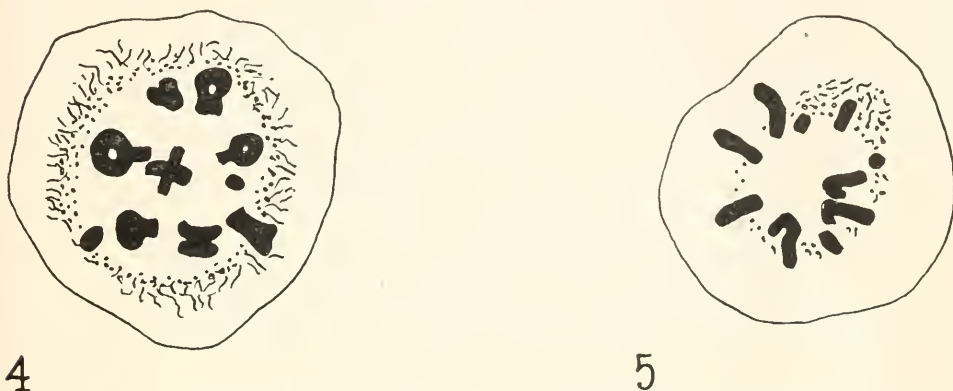
The spindle becomes visible in prometaphase through the alignment of the filamentous chondriosomes on its surface. In polar view their optical cross sections outline the spindle around its circumference (Fig. 4). In side view they appear lined up from the poles to the equator where they flow out into the equatorial plane (Fig. 1). Later, when the spindle elongates, the chondriosomes are stretched tightly on the spindle surface.

In Figure 3 the length of the spindle and its equatorial diameter are plotted against time during anaphase. We see how the spindle begins to elongate a few minutes after the onset of chromosome movement, contributing to their separation. There are thus two simultaneous processes involved in the later part of anaphase, namely, (1) the movement of the chromosomes to the poles due to the shortening of the chromosomal fibers, and (2) the elongation of the spindle. The diameter of the spindle increases in the later part of the chromosome curve, when the movement becomes irregular and slows down. Then it progressively decreases until the cleavage furrow cuts the spindle body in half. The spindle, therefore, increases

appreciably in volume during mid-anaphase. A comparison of the curves in Figure 3 shows that the spindle elongation varies more from cell to cell than the shortening of the chromosomal fibers. This indicates a greater sensitivity of that process to external conditions.

Anaphase movement in the second spermatocyte division

In the first division the chromosomes are distributed through the spindle body (Fig. 4). In the second division, however, they are oriented with their kinetochores at the periphery of a "hollow" spindle, the long arms pointing outwards (Fig. 5). Figure 6 gives the curves of three out of fifteen measured cells.² The



FIGURES 4 and 5. Camera lucida drawings of living spermatocytes of *Chorthophaga*. Polar view of primary and secondary metaphases.

movements of the chromosomes and the behavior of the spindle are much like those described for the first division. The chromosome curve is distinctly S-shaped. The spindle elongates a few minutes after the chromosomes have separated and increases in diameter in the later part of anaphase. Again there is thus a great increase in volume of the spindle. The rate of chromosome movement and spindle stretching is appreciably greater than in the first division.

In both divisions then we find the same type of anaphase movement. It begins with a shortening of the chromosomal fibers moving the chromosomes towards the poles. While this is continuing, the spindle begins to stretch, adding to the chromosome movement. Towards the end of anaphase the spindle begins to increase in width and then gradually shrinks until the cleavage furrow cuts it in half.

Experimental separation of the factors of anaphase movement

In the Hemiptera and Homoptera the action of chromosomal fibers and spindle elongation represent two distinct processes separated in time (Ris, 1943). In the grasshopper there is no such independence; the two processes are simultaneous and so neatly interwoven that a smooth movement of the chromosomes ensues. Is it possible to separate them experimentally? Methods have long been known which inhibit or destroy the spindle, such as ether, chloralhydrate, colchicine, etc. Is

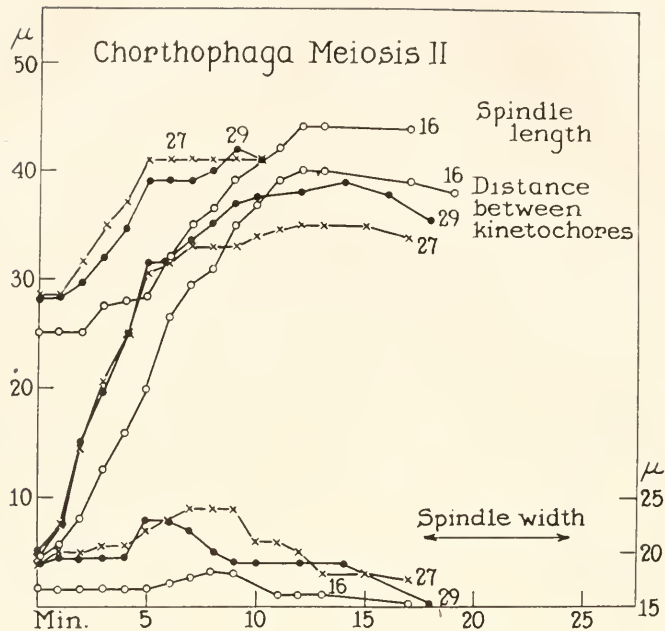


FIGURE 6. Chromosome movement and spindle behavior in the second meiotic division of *Chorthophaga viridifasciata*. See text.

there an agent which would inhibit one of the two processes without affecting the other? Colchicine, if added to the medium, either destroyed the spindle completely, or in lower concentrations had no effect on anaphase movement. Chloralhydrate, on the other hand, proved more useful. In concentrations higher than 0.1 per cent the spindle became shorter and narrower, and finally disappeared. The chromosomes were scattered irregularly through the center of the cell. The chondriosomes lost their regular orientation and began to penetrate between the chromosomes. At a concentration of 0.08 per cent cells were found in which the chromosomes moved to the poles, but where spindle elongation was inhibited. This seems to happen only within narrow limits of concentration of chloralhydrate inside the cell. If there is too much, the spindle will break down; if there is too little, it will elongate normally. This critical concentration is usually obtained only in a few cells of one cyst. The distance between chromosomes, and the length of the spindle, were then recorded during anaphase in primary spermatocytes exposed to chloralhydrate. In Figure 7 two such curves are shown (36 and 37). The spindle remained the same length all through anaphase in cell 37 and became only slightly longer in cell 36. The chromosomal fibers, on the other hand, must have remained active since the chromosomes had moved to the poles in a regular fashion. It can be shown that this action of the chromosomal fibers is normal. If we subtract the spindle elongation from the chromosome curve of an untreated primary spermatocyte, we obtain a curve which represents the movement of the chromosomes due to the chromosomal fibers alone. Two such curves are plotted in Figure 7 (13a and 17a). Since they agree well with the experimental curves (36 and 37), we must conclude that the

action of the chromosomal fibers was not affected by the chloralhydrate even though the spindle was prevented from elongating.

In the Hemiptera and Homoptera the two factors of anaphase movement, contraction of the chromosomal fibers and elongation of the spindle body, are separated in time. In the grasshopper they overlap in time, but their differing sensitivity to chloralhydrate has made it possible to separate them experimentally, inhibiting

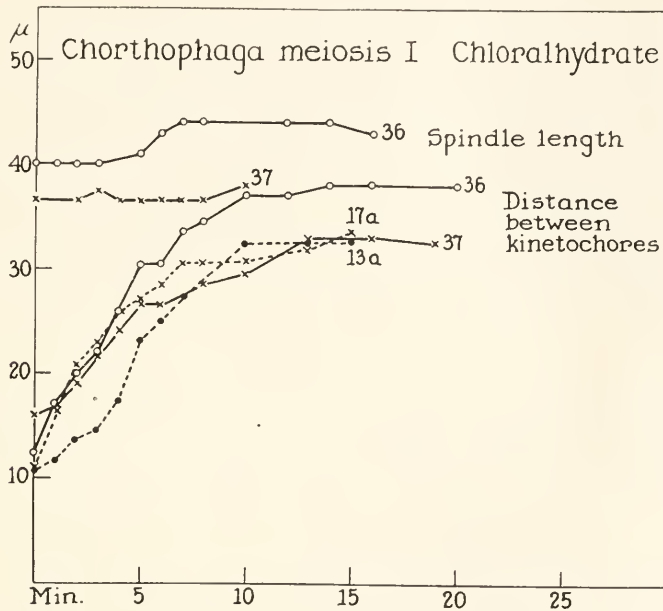


FIGURE 7. Chromosome movement and spindle elongation in Belar's solution with 0.08 per cent chloralhydrate (curves 36 and 37). Curves 13a and 17a represent the normal chromosome movement after the spindle elongation has been subtracted. The four curves are similar, showing that the movement to the poles in the absence of spindle stretching is normal.

spindle elongation without affecting the contraction of the chromosomal fibers. The difference between the grasshopper and the Hemiptera and Homoptera lies mainly in the relative timing of the component processes. In the grasshopper, spindle elongation sets in before the chromosomes have reached the poles. In the Hemiptera and Homoptera the spindle does not stretch until a few minutes after the poleward movement of the chromosomes has been completed.

Recently Callan (1941) described a case in which a separation of the components of anaphase movement occurs under natural conditions. In a trisomic grasshopper (*Mecostethus*) the unpaired extra chromosome sometimes moves into the equatorial plane during the first meiotic anaphase. In these cases the spindle does not elongate. The poleward movement of the chromosomes, however, does not seem to be disturbed. For reasons unknown, spindle elongation is inhibited under these conditions while the chromosomal fibers do not seem to be affected. Of course, we do not know here whether the rate of movement is normal as was shown in the chloralhydrate experiments.

The effect of temperature on the anaphase movement of chromosomes

The effect of temperature on mitosis has been repeatedly investigated, in most cases, however, on over-all processes such as the length of the mitotic phases, the rate of cleavage, etc. (see Belehradek, 1935). Only little can be concluded from such studies unless the processes are broken down into their components and the effect of temperature on these components analyzed. The effect of temperature on chromosome movement in living cells was studied by Bucciantie (1927) in chick fibroblasts and by Barber (1939) in *Tradescantia* stamen hair cells. They found an increase in the rate of chromosome movement with rising temperature. This increase was large at lower temperatures and small at higher temperatures. In chick fibroblasts there is a maximum rate at 40° C. Fauré-Fremiet (1925) had earlier reported an optimum temperature (37° C.) for cell division in *Ascaris*. Measurements of chromosome movement in the secondary spermatocyte of the grasshopper at 17°, 23° and 30° C. agree with these findings (Fig. 8 and Table I). The data for *Tradescantia* (Barber, 1939) and grasshopper also suggest an optimum temperature, though the range of temperature was not wide enough to show the decrease at higher temperatures. In the grasshopper a temperature above 32° C. destroys the spindle and thus inhibits chromosome movement.

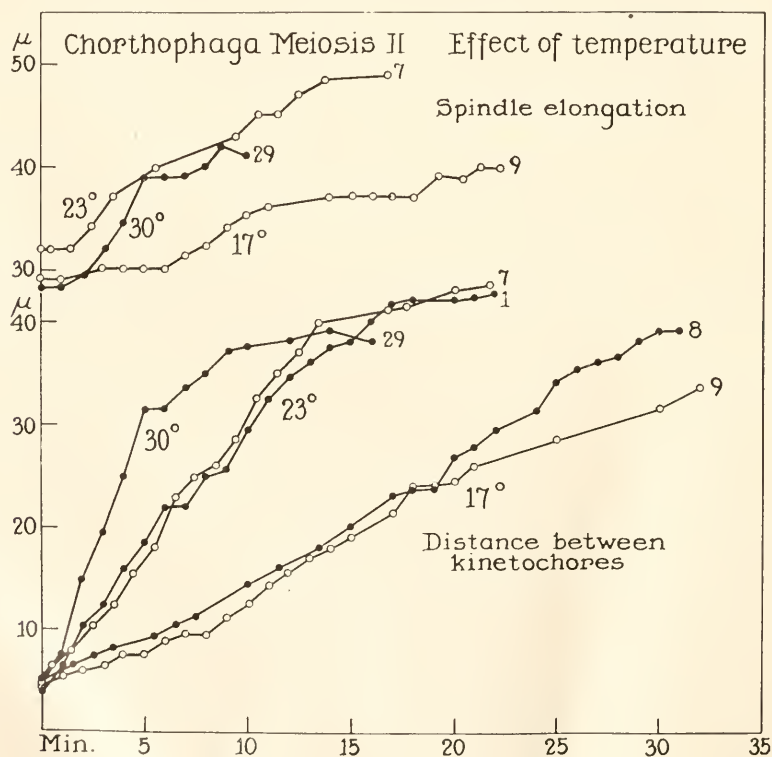


FIGURE 8. Chromosome movement and spindle elongation at 17°, 23°, and 30° C.

TABLE I

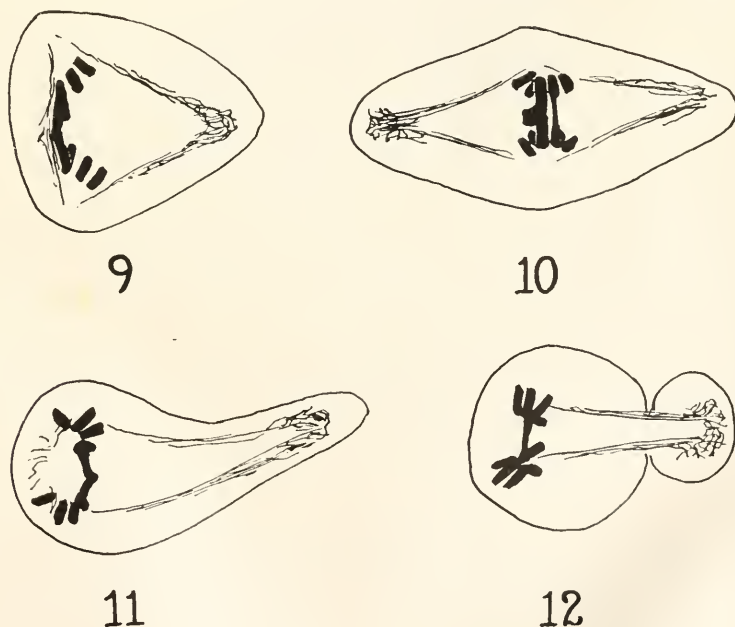
Effect of temperature on the rate of chromosome movement (chromosomal fibers only), and on the rate of spindle elongation. Micra/minute

Temperature	Maximum velocity chromosomes	Maximum rate spindle elongation
17° C.	0.4	1.4
23°	1.2	2.4
30°	2.5	3.6

Exposure to low temperature (1°–5° C.) destroys the mitotic spindle, as has been known since the experiments of O. Hertwig (1890) on sea urchins. If grasshopper spermatocytes at metaphase are exposed to 1° C., the spindle disappears, the chondriosomes become arranged at random, and the chromosomes are dispersed through the former spindle area. The cells can remain in this state for hours. If they are again exposed to a higher temperature (30° C.) the spindle forms anew, the chondriosomes are lined up on its surface and the chromosomes arranged in the metaphase plate. This process can be repeated several times on the same cells.

Abnormal spindle elongation

The increase in volume during anaphase is a characteristic property of the spindle in most animal cells. This swelling manifests itself especially in a pronounced elongation which contributes to the anaphase separation of the chromosomes that are attached to it by means of chromosomal fibers. As was shown above,



FIGURES 9-12. Diagrams demonstrating the abnormal lateral stretching of the spindle in primary spermatocytes after X-ray-induced sticking of the chromosomes.

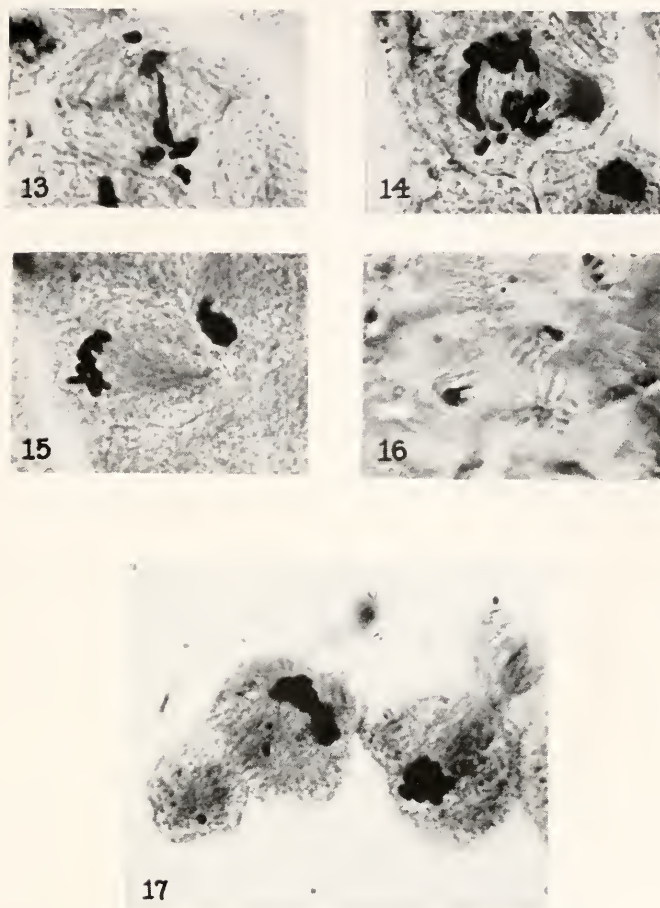
the spindle also increases in width in mid-anaphase and then gradually shrinks until it gets pinched through by the cleavage furrow. This stretching ability of the spindle is especially impressive under certain abnormal conditions. If the first meiotic division is observed in living cells after X-raying, in hypertonic medium or at temperatures around 32° C., one finds that chiasmata have a tendency to stick so that bivalents can not separate at anaphase. When the spindle begins to stretch, its normal elongation in the polar axis is inhibited by the combination of chromosomal fibers and sticking chromosomes. The spindle then begins to bulge in the equator, opposite the sticking chromosomes (Figs. 9, 14). If several bivalents fail to separate, the spindle may bend outward in several places (Figs. 10, 16). With the further elongation of the spindle, these lateral bulges become long and narrow projections which begin to push out the cell membrane. The poles of the spindle approach each other during this process, probably because of the action of the chromosomal fibers, which, instead of pulling the chromosomes to the poles, now draw the poles closer together (Figs. 11, 15). In fixed and stained preparations, the course of the continuous fibers shows clearly that the lateral projections are parts of the spindle bending outwards (Figs. 13-15). If chromosomes stick only on one side of the spindle, a very characteristic bent spindle results, looking like a spindle folded in the middle. Actually the origin is quite different, as described above. Sometimes the sticking chromosomes separate in mid-anaphase. The spindle then is able to assume its normal shape. It elongates in the polar axis and the lateral bulges disappear.

Spindle elongation and cleavage furrow

The relation of spindle elongation to cytoplasmic division, as demonstrated by these abnormal anaphases, is of special interest. When the spindle does not elongate normally, the cleavage furrow is always delayed or does not appear at all. More striking are the cells in which the spindle has been forced to elongate laterally in the equatorial plane. The lateral bulges of the spindle begin to push the cell out into long narrow processes (Figs. 11, 14). At the time when normally the cleavage furrow is formed, constrictions become visible around these cell projections. Often these constrictions develop into regular cleavage furrows and pinch off one or more small anuclear buds (Figs. 12, 17). In the cysts with secondary spermatocytes, one finds then cells with the diploid number of chromosomes which undergo the second division, and anuclear buds which do not divide any more. In grasshopper spermatocytes the cleavage furrow is therefore dependent on cell elongation caused by the stretching of the spindle. The location of the furrow is not predetermined, but can occur wherever the cell is pushed out.

Time relations

The main difficulty in the timing of the phases of mitosis, particularly in living cells, is the separation of the process into clearly delimited sections. The usual separation into prophase, metaphase, anaphase and telophase is not well suited for this purpose since the beginning or end of these phases is usually without sharp boundary. The duration of the following well-marked phases was measured in the spermatocytes of the grasshopper at 30° C.: First division: (1) Metakinesis, from



FIGURES 13-15. Anaphase in primary spermatocytes of *Chorthophaga* after irradiation with X-rays (100 r). Note the sticking of chromosomes and the lateral expansion of the spindle. Fixation: Sanfelice; stain: Iron-hematoxylin. 4 mm. Zeiss Apochromat, 15 $\times$ ocular. Compare with Figures 9-12.

the disappearance of the nuclear membrane to the formation of the metaphase plate. (2) Metaphase, from the establishment of the metaphase plate to the beginning of anaphase separation. (3) Ana-telophase, from the beginning of chromosome (kinetochore) movement to the appearance of the nuclear membrane. Interphase: from the formation of the nuclear membrane to its breakdown in the secondary spermatocyte. Second division: same phases as in the first division. The processes which mark these stages are clearly visible in the living cell. In the first division the asters are visible mainly due to the radial arrangement of the chondriosomes, but sometimes astral rays can be seen. The nuclear membrane, which was sharply outlined in prophase, becomes irregular and wrinkled, then disappears first near the asters. Wrinkled remnants can be seen for a few minutes before they vanish.

The metaphase spindle then slowly takes shape after the orientation of the asters. Its outline is marked by the chondriosomes. The spindle is at first rather narrow and short (cf. Belar, 1929, p 433). The chromosomes are thus crowded into the middle of the cell. Then, while the chromosomes become arranged into the metaphase plate, the spindle increases in width and in length. During metaphase the spindle remains constant in size and varies little from cell to cell. In telophase the nuclear membrane appears around a light area containing the chromosomes. The nucleus then enlarges until the regular interphase size is reached. In the second division the same processes are repeated, except that no asters can be seen in living cells. Table II gives the duration of these phases. Cells 1 and 2 were followed through the two divisions, cells 3 and 4 only through part of meiosis. Cells 3 and 4 are from a different individual than cells 1 and 2. At constant temperature the length of each phase varies only slightly from one individual to another.

TABLE II

Time relations in the meiotic divisions of the grasshopper Chorthophaga (30° C.) (hours and minutes)

	Cell 1	Cell 2	Cell 3	Cell 4
I. Metakinesis	50	—	43	—
I. Metaphase	2/30	2/45	2/40	—
I. Anaphase	1/25	1/40	1/25	—
Telophase				
Interphase	2/15	2/40	—	—
II. Metakinesis	25	—	—	25
II. Metaphase	1/25	1/25	—	1/35
II. Anaphase	1/45	1/47	—	1/45
Telophase				

DISCUSSION

The causal analysis of mitosis strives to dissect the complex process of cell division into its component factors and to elucidate their composition and their mode of action. Since Belar's classical study, the structures involved in the mitotic movements have again received deserved attention. In the grasshopper we can distinguish the following mitotic organelles: center, kinetochores, chromosomal fibers, spindle body.

The center

Like most animal cells the grasshopper spermatocytes contain a pair of centrioles which move to opposite sides of the nucleus in prometaphase and form the poles of the developing spindle. The asters are rather inconspicuous as in other cells with relatively little cytoplasm. In living cells they can be seen in prophase and occasionally in metaphase, especially in a hypertonic medium (cf. Belar, 1929). In secondary spermatocytes asters are even less distinct. Little is known about the function of the centers, except that they are probably involved in the organization of the spindle and in the cytoplasmic streaming which goes on during metaphase and anaphase.

Kinetochores

This specialized region of the chromosome is essential for the regular movements within the spindle. Fragments which are devoid of it lag behind and do not show any regular orientation, but may be moved passively by the stretching spindle or cytoplasmic currents on the surface of the spindle (White, 1935, 1937; Carlson, 1938). The main function of the kinetochores, perhaps in cooperation with the spindle or centriole, appears to be the formation of chromosomal fibers. Without kinetochores no chromosomal fibers can be formed.

Chromosomal fibers

Soon after the nuclear membrane has disappeared and the spindle begins to take shape, we can find in fixed and stained cells a distinct fibrous connection between the kinetochores and the spindle poles. These are the chromosomal fibers. They are usually not visible in living cells and some investigators therefore deny their existence.³ Yet there is enough circumstantial evidence to show that they exist as differentiated structures within the spindle, and that they are the major factor in the anaphase movement of chromosomes (cf. Cornman, 1944; Schrader, 1944).

Belar (1929) emphasized the role of these "traction fibers." He assumes that they originate as a fluid secretion by which the kinetochore attaches itself to a fiber of the spindle body ("Leitfaser") and which allows the chromosome to glide along this "Leitfaser" in anaphase. Schrader (1944) accepts this view of Belar and bases on it his classification of spindles. Yet, the present writer could find no evidence for this indirect formation of the chromosomal fibers. They appear in pro-metaphase even before the spindle is fully formed as direct connections to the centers. They anchor the chromosomes to the poles of the spindle. So, when the spindle elongates, the chromosomes are carried along, the pull being transmitted through the chromosomal fibers to the kinetochores. If a chromosome sticks at anaphase, it will prevent the spindle from elongating on that side. The combination chromosome-chromosomal fibers is thus stronger than the spindle, while the cell membrane, for instance, yields to its pushing force. The chromosomal fibers, when they contract at anaphase, can even pull the spindle poles together and force the spindle out to one side of the cell (Figs. 9-12). The chromosomal fibers must thus be of greater consistency than the spindle body.

The spindle body

In grasshopper spermatocytes the spindle body develops from nuclear material between the two centers. The area around the chromosomes remains distinct even after the nuclear membrane has disappeared, and stays free of cytoplasmic inclusions like chondriosomes. In metaphase the spindle is a viscous body which can be moved about and dissected out by microneedles (Chambers, 1924). It appears homogenous in the living cell and fibrous after fixation. The spindle is essential for the orientation of chromosomes and for the action of chromosomal fibers since they are anchored at its poles. The spindle can be destroyed by a number of agents: colchi-

³ Chromosomal fibers are sometimes visible in forms with diffuse kinetochore, if the chromosomes are viewed on end and the light therefore has to pass the entire length of the sheet-like chromosomal fibers (Hughes-Schrader and Ris, 1941; Ris, 1942).

cine, chloralhydrate, cold, heat, hypertonic medium, etc. At the same time the regular arrangement of the chromosomes disappears and all chromosome movements are stopped.

The most striking action of the spindle is the elongation during anaphase. Belar (1929) found that in hypertonic media this elongation appears to be greatly exaggerated, and this led him to a very detailed study of spindle stretching in hypertonic solutions. His conclusions are briefly: (1) The spindle has a tendency to stretch; this tendency is exaggerated in hypertonic media. (2) The spindle, by origin, is differentiated into two half spindles, the "Stemmkörper" (pushing body) developing at anaphase between the daughter plates. (3) At anaphase, it is the "Stemmkörper" in particular which elongates.

As was shown above, abnormal spindle stretching occurs not only in dehydrated cells, but always when daughter chromosomes are made to stick together. A hypertonic medium is just one way of causing chromosomes to stick at anaphase. This effect of hypertonic solutions on chromosomes was described by Konopacki (1911) in cleavage divisions of echinoderm eggs, by Kostanecki (1898) in *Myzostoma*, and by Moellendorff (1938) in tissue cultures. Similar accidents are found at high temperatures (over 30° C.) and after exposure to X-rays. It is therefore not the hypertonic medium which induces the abnormal spindle stretching, but the resistance to elongation in the main axis, brought about by the sticking of chromosomes. From Belar's figures, it is obvious that in primary spermatocytes all the abnormal spindles are correlated with sticking chromosomes. The bent spindles in secondary spermatocytes are of a different and less extreme kind (Fig. 45, Belar, 1929). Here it seems to be the cell membrane which offers resistance to the elongating spindle and causes it to bend. X-ray-induced bridges cause the same kind of abnormal spindles in secondary spermatocytes as Belar described in the first division. Lateral expansion of the spindle after X-ray-induced chromosome sticking was also figured by White (1937, Figs. 12, 13).

But Belar figures some cells which show exaggerated elongation of the spindle without sticking of chromosomes. These cells had been treated in anaphase. During anaphase the spindle increases not only in length, but also in volume. Belar believed that the volume remained constant, though he did not commit himself definitely. His beautiful drawings, however, indicate quite clearly the swelling of the spindle which measurements have now substantiated. In hypertonic solutions the cell shrinks greatly, and as Belar pointed out, the cytoplasm more so than the spindle. The swelling of the spindle, then, encounters resistance, and it is probably this factor which causes the spindle to be longer, but narrower than normally. Even so, these spindles are only found in free floating cells. In this writer's preparations where the cells remained in the follicles, they did not occur. Another case of spindle stretching without chromosome sticking is found if prometaphases are treated with hypertonic solutions (Belar, 1929, Fig. 55; Ris, 1942, in spermatocytes of the bearberry aphid). Here again the spindle increases in width during its formation. In dehydrated cells this lateral growth is interfered with, and the spindle becomes long and narrow. In this connection it is important to note that the volume of the abnormally stretched spindles appears to be not larger than in normal spindles. There is, therefore, only a distortion in shape, not an actual increase in the spindle material.

Two factors then cause abnormal spindles: interference with the increase in width in prometaphase and mid-anaphase, and interference with normal stretching during anaphase through the sticking of chromosomes.

From his studies of these abnormal spindles, Belar came to the conclusion that the part of the spindle between the separating chromosomes was mainly responsible for the stretching. He called it the "Stemmkörper" (pushing body) and distinguished it from the two half spindles between the chromosomes and poles. This subdivision of the spindle is, however, artificial and unjustified. Belar himself points out the uniformity in the aspect of the entire spindle. Fibers and clefts are continuous. The only difference in anaphase is the presence of chromosomal fibers in the cone-shaped region between the chromosomes and the poles. This is responsible for the darker appearance after staining. In Belar's Figure 41 the chromosomal fibers are especially clear. The "Stemmkörper" concept originated in the observation that the region between the daughter plates elongates more rapidly than the entire spindle. This appears so, not because this region is a special part of the spindle, but because the chromosomal fibers actually shorten during spindle elongation, pulling the chromosomes to the poles. In this way the impression of a special stem body between the daughter plates is produced.

Furthermore, Belar thought that the initial separation of the chromosomes through action of the traction fibers releases the tension in the spindle and originates the action of the "Stemmkörper." But actual timing has now shown that the chromosomes travel a good distance to the poles before the spindle elongates. Besides, spindle stretching can occur without any action of the chromosomal fibers as is shown in the first spermatocyte division of *Tamalia*. The chromosomal fibers act merely as passive anchors for the chromosomes (Ris, 1943). In the lepidopteran *Orgyia* the spindle elongates though the chromosomes have no chromosomal fibers at all (Cretschmar, 1928, Figs. 48-50). All the evidence then indicates that there is no differentiation into "half spindles" and "Stemmkörper." The only real differentiations are the chromosomal fibers and the spindle body. The chromosomal fibers pull the chromosomes to the poles. The stretching of the spindle has nothing to do with this phase. It can go on just as well without spindle elongation (chloralhydrate experiment). But spindle elongation has its important functions. It separates the daughter plates still further by pushing the poles apart and thus indirectly moves the chromosomes anchored to them.

The picture of anaphase movement in grasshopper spermatocytes presented here is essentially in agreement with Belar's view. There is a "pulling action" of chromosomal fibers and the stretching of the spindle. But there are some modifications. The subdivision into half spindles and "Stemmkörper" is found to be artificial. The spindle as a whole elongates, at the same time increasing in volume. Its action on the chromosomes is indirect, through the chromosomal fibers which connect them to the spindle poles.⁴ The chromosomal fibers are thought to connect the kinetochores directly to the poles without the intervention of a "Leitfaser." They shorten during anaphase and are alone responsible for moving the chromosomes to the spindle poles.

⁴ Just how the chromosomal fibers are attached to the spindle is a very puzzling problem and nothing definite can be said about it at present.

In addition to being a major factor in the movement of chromosomes, the spindle body also seems to play a role in the division of the cytoplasm. If the spindle does not elongate, as in the chloralhydrate experiments, no cleavage furrow is formed. When the spindle stretches laterally instead of in its long axis, a cleavage furrow does appear at a right angle to this elongation in a quite unorthodox position and produces an anuclear bud (Figs. 9-12). Bauer (1931) illustrates a similar situation in spermatocytes of *Tipula* with abnormal spindles. His Figure 23 h suggests that it originated in the same fashion. Many examples can be found in the literature which show how the failure of spindle stretching causes absence of the cleavage furrow (for instance Dobzhansky, 1934; Callan, 1941). In most plant cells there is little or no stretching of the spindle and the cytoplasm is divided by the formation of a cell plate. But in the pollen mother cells of some plants a cleavage furrow is formed, and it is then associated with elongation of the spindle (Guignard, 1897; Farr, 1918). It appears then that in dividing cells, elongation of the cell and cleavage furrow are associated with spindle elongation (in contradiction to the unwarrantable generalization of Buchsbaum and Williamson, 1943). Dan has recently (1943) assembled convincing evidence that spindle elongation is the active agent in cell elongation and the following formation of a cleavage furrow.⁵ On the other hand, in certain abnormal cases the spindle elongates and yet no cleavage furrow appears. The formation of a cleavage furrow clearly depends on other factors in addition to spindle elongation.

In a recent paper, Hughes and Swann (1948) published chromosome separation and spindle elongation curves for chick embryo cells in tissue culture. Chick chromosomes possess a localized kinetochore and the achromatic apparatus is similar to that of the grasshopper spermatocytes. The anaphase movement as described by the curves of Hughes and Swann is essentially the same as we found in the grasshopper. Their curves show spindle elongation to start right from the beginning of anaphase, while in the grasshopper it does not begin until the chromosomes have moved a considerable distance. This is probably not a real difference but the result of the great difficulties involved in making measurements in early anaphase on the small chromosomes and spindles of the chick embryo cells.

We have set out to describe the movement of chromosomes during anaphase in terms of the mitotic organelles involved. The structures responsible for this chromosome movement were found to be the chromosomal fibers and the spindle body. The chromosomal fibers move the chromosomes to the spindle poles by decreasing in length. The spindle body swells and stretches and moves the daughter chromosomes further apart, since they are anchored to the spindle by the chromosomal fibers. If these are broad sheets attached to the entire length of the chromosome (diffuse kinetochore), the spindle does not elongate until the chromosomes have reached the spindle poles (hemipteran and homopteran insects). If the chromosomal fibers are narrow bundles attached to a very short region of the chromosome (localized kinetochore), the spindle begins to stretch shortly after the chromosomes have begun to move. The two processes then act simultaneously producing a smooth unbroken chromosome separation curve. Though we cannot see here directly how the two factors act on the chromosomes, we can separate them experi-

⁵ I am indebted to Dr. D. Costello, University of North Carolina, for making this paper available to me.

mentally by inhibiting spindle elongation with chloralhydrate. It is then possible to study the action of the chromosomal fibers alone.

Very little is known about the nature and mode of action of these organelles, and this aspect will not be discussed here. Many more exact data on the structure, composition, and behavior of spindle and spindle components under various conditions are needed before a fruitful hypothesis on the physico-chemical level can be brought forward.

SUMMARY

The movement of chromosomes and the changes in spindle size have been recorded in living spermatocytes of the grasshopper during the meiotic divisions. Anaphase movement consists of two separate processes which are related to the action of distinct cellular organelles: (1) The shortening of chromosomal fibers moves the chromosomes to the poles. (2) The elongation of the spindle further separates the daughter plates. The two processes act simultaneously in the grasshopper. With chloralhydrate, spindle elongation can be inhibited without affecting the action of the chromosomal fibers. This demonstrates the independence of these two factors.

The effect of temperature on chromosome movement is shown by measurements at 17°, 23° and 30° C. Between 17° and 23° there is a greater increase in velocity of chromosome movement than from 23°–30° C. Temperatures above 32° C. inhibit mitosis through the destruction of the spindle.

Abnormal spindle elongation is found whenever chromosomes stick at anaphase. The spindle, unable to elongate in its long axis, expands laterally into a disc-shaped body which later forms one or several finger-like processes, pushing out the cell membrane. These lateral elongations usually give rise to one or more cleavage furrows, pinching off one or, rarely, more anuclear buds. This demonstrates clearly the relationship between spindle elongation, cell elongation, and cleavage furrow.

The role of the mitotic organelles in the anaphase movement of chromosomes is discussed. Indispensable for a regular anaphase are the kinetochores on the chromosomes, the chromosomal fibers, and the spindle body. No evidence was found for a specialized region in the spindle acting as "Stenmkörper." The spindle is uniform in structure and elongates uniformly.

Distinct recognition of the structures involved in anaphase movement, and a quantitative description of their function, forms a basis for experimental analysis of their composition as well as their mode of action.

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THE BIOLOGICAL BULLETIN

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TEMPERATURE COEFFICIENTS OF RESPIRATION IN PSAMMECHINUS EGGS

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INTRODUCTION

In *Arbacia punctulata* Rubenstein and Gerard (1934) using Warburg technique, found a Q_{10} of oxygen consumption for fertilized eggs of 1.8 between 13 and 30° C., whereas unfertilized eggs had a much higher value, viz., 4.1. These remarkable findings were principally confirmed by Korr (1937), who extended the experiments (Warburg technique) and discussed the results from biochemical and physiological points of view.

On the other hand Tyler and Humason (1937), working on *Strongylocentrotus purpuratus* (Warburg technique), found no significant difference in respiration Q_{10} between fertilized and unfertilized eggs in the temperature range 5–22° C. So, for example, the Q_{10} values for 10–20° C. were 2.3 and 2.6 respectively. Similar results were reported for *Dendraster*, *Ciona* and *Urechis*.

In earlier investigations (Warburg technique) by Ephrussi (1933) on *Paracentrotus lividus* the same Q_{10} of oxygen uptake was found between 14.9 and 22.4° for unfertilized eggs and gastrulae, viz., ~ 2 –2.5. At lower temperatures there was a tendency to slightly higher Q_{10} values in the eggs. Loeb and Wasteneys (1911) using Winkler technique reported very low values for unfertilized *Arbacia* eggs ($Q_{10} \sim 1.3$ between 5 and 25° C.), but normal values for fertilized eggs ($Q_{10} \sim 2$ –2.5 for the range 3–25° C.).

In view of the probable significance of temperature coefficients of respiration for elucidating the different oxidative mechanisms of fertilized and of unfertilized sea-urchin eggs, it was thought of importance to investigate the matter in another species. Recently Borei (1948a) studied the respiration of eggs of *Psammechinus miliaris* before and after fertilization. Because of the facts already known about this species, it was chosen for the present investigation.

EXPERIMENTAL DATA

The experiments have been performed with Cartesian diver micro-respiration technique, suitable for measurement of the oxygen consumption of ~ 100 eggs at a time. Concerning material and methods, corresponding chapters (2.1-2.3; 3.111; 3.114) in Borei (1948a) should be consulted. Diver charge type I (Borei, 1948b) was used throughout.

The temperature range was 10-21° C. The maximum temperature for normal larval development of *Psammocchinus miliaris* has been studied by S. Runnström (1927), who found it to be 22° C.; the minimum temperature was found by this author to be 8° C. For measurements at lower temperatures, the cooling coil of the diver apparatus thermostat was fed with refrigerated salt-water of approximately +5° C. The desired temperature was obtained by counteracting the cooling device by operating a thermostatically controlled electric heating bulb.

In order to obtain more comparable values, measurements were only performed on the flatter part of the declining respiration curve of the unfertilized egg (cf. Borei, 1948a, Chapter 3.112.1). Thus the time of actual measurement usually ran from three to six hours after removal from the ovary. This means that the constant part of the respiration is dominant during the measurements, whereas the "rapidly declining" part characterizes the preceding 2.5-3 hours, during which the eggs were kept at 16-18° C. The eggs were, on an average, placed in the diver 2.5 hours after removal from the ovary. The diver was then immediately placed in the thermostat at the experimental temperature and left there for a half hour for temperature equilibration before starting the measurements. Usually two diver thermostats were operated simultaneously, thus allowing measurements at two different temperatures. The time schedule of the experiments may be seen from Figure 1.

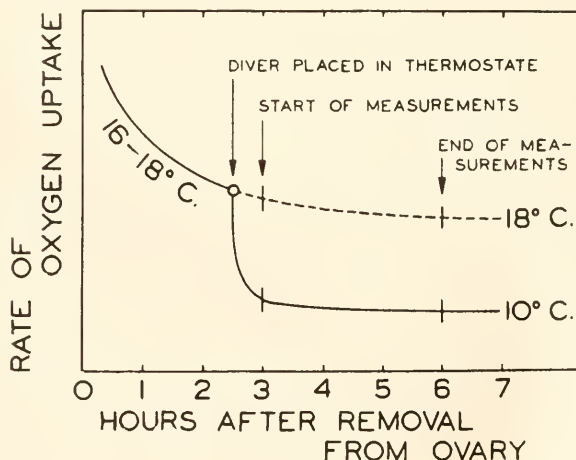


FIGURE 1. Average time schedule of experiments.

An actual experiment with measurements at 10° C. and a control experiment at 18° C. is assumed in the figure.

After completed diver measurements, the cells were washed out of the divers with sea-water, re-counted and then microscopically observed as to condition and fertilizability. Only those experiments were accepted in which the cells passed these post-diver measurement controls satisfactorily.

Previous authors have stated their results in rates of oxygen consumption per volume of cell matter. Great pains were taken by them to estimate the volume accurately. Probably the most correct way will be that adopted by Korr (1937): egg volume obtained by multiplying the number of eggs by the average egg volume. Technical difficulties were met, however, in estimating the exact number of eggs. In the present investigation neither the counting of the eggs nor the measurement of the average size of the eggs will give any difficulties. In view of these facts and of the great variability of the cell diameter, it has been thought more advisable, even in this investigation, to state the rate of oxygen uptake on a cell volume basis. This procedure also permits of direct comparison with the results of the previous authors.

In order to obtain a measure of the cell volume, the cell diameter was estimated of a number of eggs (> 20) from every female used, by means of a calibrated ocular micrometer (cf. Borei, 1948a, Chapter 3.114).

TABLE I

Oxygen consumption at different temperatures of unfertilized Psammechinus miliaris eggs

Volume of oxygen, measured at 0° C. and 760 mm. Hg, consumed per volume of cell matter and hour. All measurements are on egg material from S-form animals, except those marked *, which are on material of the Z-form.

Temperature (° C)	Oxygen consumption	Temperature (° C)	Oxygen consumption	
10	0.024	18	0.080	
	0.053		0.082	
	0.069		0.084	
11	0.062		0.099	
	0.066		0.107*	
	0.069		0.132	
12	0.067		0.151*	
			0.160	
13	0.056	19	0.086	
	0.076		0.089	
14	0.078		0.140	
	0.083		0.145	
15	0.060		0.173	
	0.113		0.188	
16	0.033	20	0.065	
	0.046		0.111	
	0.099		0.116	
	0.112		0.126	
17	0.061	21	0.090	
	0.076		0.122*	
	0.088		0.131	
18	0.063		0.172*	
	0.065			

The oxygen consumption figures obtained are referred to 0° C. and 760 mm. Hg, in order to render them intercomparable, irrespective of the actual temperature of measurement. Corrections are introduced according to Figure 2 of Borei (1948a) for deviations from the time schedule of the above Figure 1.

The results of the experiments are given in Table I.

The average cell volume, calculated from the separate figures used for the evaluations in Table I, is 5.89×10^{-4} μ l. per egg ($n=44$). (Corresponding value given by Borei, 1948a, = 5.84×10^{-4} .) The average oxygen consumption rate at 18° C. was found in the experiments ($n=10$) to be 0.51×10^{-1} μ l. per cell and hour. (Corresponding value given by Borei, 1948a, = 0.53×10^{-1} .)

DISCUSSION

Rubenstein and Gerard (1934) expressed their results according to the van't Hoff-Arrhenius equation and thought that the critical thermal increments (μ) might indicate the nature of the oxidative processes of the unfertilized and fertilized egg. This view was criticized by Korr (1937), who stressed that biological scattering and the narrow temperature limits within which respiration can be measured make the graphical evaluation of μ -values uncertain. Moreover, biological processes are governed by enzyme reactions. Such reactions have repeatedly been found not to give constant thermal increments.

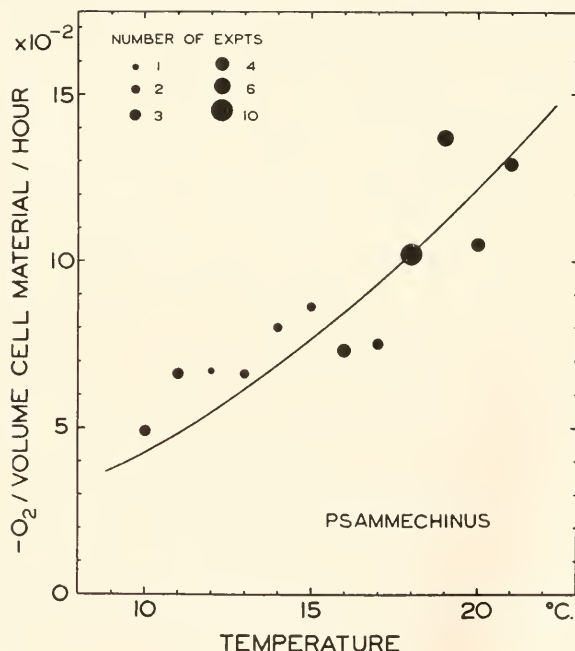


FIGURE 2. Oxygen consumption at different temperatures of unfertilized *Psammechinus miliaris* eggs.

Each dot is the mean value for the temperature in question. Diameter of dot indicates number of experiments. A standard curve according to Krogh (1914) is drawn in, passing through the value of 18° C.

The mass plot, recommended by Korr (1937), is difficult to interpret owing to the extent of the biological scattering. The mean of the values at each temperature gives a better representation. Figure 2 is plotted in this manner.

The figure shows that the standard curve of Krogh (1914) describes the obtained results quite well. This curve was originally obtained in basal metabolism experiments and found to be valid for a number of vertebrates. It was recently extended by Zeuthen (1947) to hold even for the respiration of a number of minute, chiefly marine invertebrate organisms. Thus Krogh's standard curve better describes the response in respiration on increase of temperature than does the van't Hoff-Arrhenius equation.

Formula (1)

For the temperature range of the present investigation the curve indicates that the relative temperature increment is very closely proportional to the relative increment in respiration. Thus it may be expressed by $(R_1/R_2)(t_2/t_1) = \text{const.}$, where R_1 and R_2 are the rates of respiration at the temperatures t_1 and t_2 . The constant is dependent on the chosen difference between t_1 and t_2 . For $t_1 - t_2 = 2^\circ \text{C.}$, it is = 0.93.

In Figure 3 the results of this and previous investigations are given on a relative scale. The curve according to Formula (1) represents the material of this investigation. With this curve coincide the values of Tyler and Humason (1937) on the respiration of *Strongylocentrotus* eggs. On the other hand the results with *Arbacia* differ markedly from those on *Psammechinus* or *Strongylocentrotus*.

The temperature characteristics of the unfertilized sea-urchin egg thus represent two distinct classes: (1) the *Arbacia* type with high Q_{10} values, and (2) the *Strongylocentrotus*-*Psammechinus* type with Q_{10} values in close concordance with the standard curve of Krogh.

The temperature coefficients of the fertilized *Psammechinus* egg differ in no way from those found by previous authors for fertilized eggs of other sea-urchin species. Thus in the range $12-20^\circ \text{C.}$ a $Q_{10} \sim 2-2.5$ was found.

A comparison of the Q_{10} values found in different investigations further stresses that there are two classes in respect to temperature characteristics of respiration of the unfertilized eggs (see Table II). *Strongylocentrotus* and *Psammechinus* have for both fertilized and unfertilized eggs a Q_{10} value at room temperature of approximately 2.5. The fertilized *Arbacia* egg shows the same value, but the value of the unfertilized egg is higher. The unfertilized egg of *Paracentrotus* has, at lower temperatures, a tendency in the same direction as the *Arbacia* egg.

Apparently Q_{10} as in most biological processes is higher at lower temperatures. The results of Tyler and Humason (1937) and of Korr (1937) seem, however, to contradict this conclusion, but the aberrations are probably to be attributed to experimental circumstances. (Lucké and co-workers, 1931, found that the Q_{10} of the permeability of the *Arbacia* egg to water increased with temperature.)

In the sea-urchin egg the rate of respiration is increased greatly by fertilization. This higher respiration is suppressed by cyanide, CO and other poisons of cytochrome oxidase. The oxidase in operation is an iron porphyrin, but probably not fully identical with the usual cytochrome oxidase. The respiration of the unfertilized egg was found by Ruunström (1930) and Korr (1937) to be comparatively

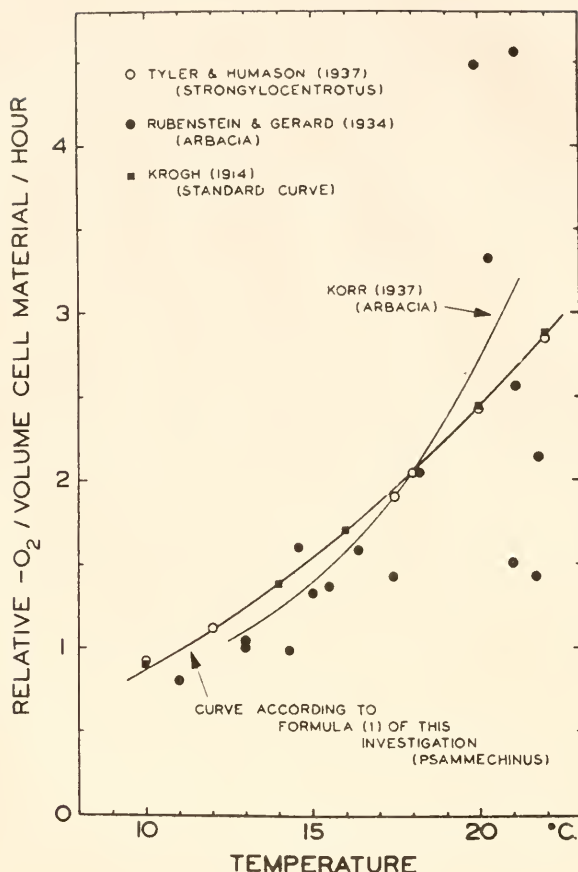


FIGURE 3. Comparison between different authors' results concerning temperature dependency of oxygen consumption in unfertilized sea-urchin eggs.

Rates of oxygen consumption given on a relative scale, putting the values at 18° C. alike. The curve for Korr's results is recalculated from this author's Figure 8.

slightly affected by said oxidase poisons. Both oxidase and dehydrogenases can be brought to work as effectively in the unfertilized egg as in the fertilized one. This permits the conclusion that neither the oxidase nor the dehydrogenases are the limiting factors in the respiration of the unfertilized egg (Runnström, 1935, 1942; cf., however, Ballentine, 1940). Runnström thinks that the difference in respiration rate between unfertilized and fertilized eggs is caused in the unfertilized egg by a lack in substrate saturation of the oxidase. Korr interprets the difference as being dependent on a factor in the oxidase part of the carrier chain, inactive or held apart in the unfertilized egg, but put into operation on fertilization. He thinks that this link might be cytochrome c and furthermore that the unfertilized egg respire over an antioxi-dizable, non-ferrous carrier. For a fuller review see Needham (1942) and Borei (1948a).

TABLE II

Q₁₀ values of oxygen consumption of sea-urchin eggs

Author	Unfertilized eggs	Fertilized eggs
Loeb and Wasteneys (1911) †	<i>Arbacia punctulata</i> 5–25° C. $Q_{10}=1.31$)§	<i>Arbacia punctulata</i> (round 1st mitosis) 3–27° C. $Q_{10}\sim 2-2.5$
Rubenstein and Gerard (1934) *	<i>Arbacia punctulata</i> 13–30° C. $Q_{10}=4.1$	<i>Arbacia punctulata</i> (up to 5 hours after fert.?) 13–30° C. $Q_{10}=1.8$
Korr (1937) *	<i>Arbacia punctulata</i> 13–23° C. $Q_{10}=3.7$ 18–28 4.5	<i>Arbacia punctulata</i> (up to 5 hours after fert.?) 13–23° C. $Q_{10}=2.2, 2.8$ 18–28 2.5, 3.1
Ephrussi (1933) *	<i>Paracentrotus lividus</i> 10.4–22.4° C. $Q_{10}=3.85$ 12.9–22.4 3.28 14.9–22.4 2.18 16.75–22.4 1.88	<i>Paracentrotus lividus</i> (gastrulae) 10.4–22.4° C. $Q_{10}=2.36$ 12.9–22.4 2.57 14.9–22.4 2.32 16.75–22.4 1.88
Tyler (1936) *		<i>Strongylocentrotus purpuratus</i> (round 1st mitosis) 7.5–20° C. $Q_{10}=2.54$ 10 –20 2.30 15 –20 1.85 (25th–26th hour after fert.) 15 –20° C. $Q_{10}=1.88$
Tyler and Humason (1937) *	<i>Strongylocentrotus purpuratus</i> 7.5–17° C. $Q_{10}=2.67$ 8 –18 2.58 10 –20 2.63 12 –22 2.54 5 –20 2.35	<i>Strongylocentrotus purpuratus</i> (a few hours after fert.) 7.5–17° C. $Q_{10}=2.79$ 8 –18 2.69 10 –20 2.26 12 –22 2.33 5 –20 2.62
This investigation †	<i>Psammechinus miliaris</i> 10–12° C. $Q_{10}=3.52$ 12–14 3.07 14–16 2.81 16–18 2.58 18–20 2.40 20–22 2.29	<i>Psammechinus miliaris</i> (a few hours after fert.) 12–20° C. $Q_{10}\sim 2-2.5$

† Winkler technique.

‡ Diver technique.

* Warburg experiments.

§ Only a single experiment.

Recent investigations on *Arbacia* eggs (Robbie, 1946) show that the respiration of the unfertilized egg can also be completely abolished by cyanide. A cyanide-stable respiration, catalyzed by formed CN-compounds, develops, however, after the initial inhibition. These findings confirm Lindahl's (1939, 1940 and 1941) results, and are in full concordance with his opinion that the cyanide-resistant respiration develops under the influence of the cyanide and that it has nothing to do with normal respiration. Robbie's findings show that the respiration of the unfertilized egg must proceed over iron porphyrins in the same manner as does that of the fertilized egg. Runnström (1930) had previously expressed the same opinion.

Robbie's results show conclusively that the respiration is of principally the same type both before and after fertilization. Thus it is understandable that temperature characteristics of respiration are found, as in the *Psammechinus* and *Strongylocentrotus* cases, that are the same before and after fertilization. On the other hand it is harder to understand how Q_{10} can differ so widely before and after fertilization as it does in *Arbacia*. It may be that the respiratory system operating in the unfertilized *Arbacia* egg is somehow unlike that of the just mentioned species. Any fundamental respiration pattern differences between the two classes of unfertilized sea-urchin eggs have, however, not been found.

Ballentine (1940) thinks that the oxidation in the sea-urchin egg of dimethyl-p-phenylenediamine requires cytochrome c as a mediator to the echinoderm oxidase. If so, cytochrome c must already be available and ready to function in the unfertilized egg, since Runnström (1932) and Örström (1932) found that dimethyl-p-phenylenediamine was oxidized at the same rate in unfertilized and fertilized eggs. Borei and Renvall (1949) could, however, not find that cytochrome c is essential for the cellular oxidation of dimethyl-p-phenylenediamine. Furthermore, hydroquinone, which can be oxidized by cytochrome oxidase (Keilin and Hartree, 1938) or echinoderm oxidase (Krahl and co-workers, 1941; Borei, 1945, Chapter IV: C1) only in the presence of a suitable mediator, has been shown by Runnström (1932) to be utilized in the unfertilized egg at a lower rate than in the fertilized one. This fact could possibly be taken as an indication that the carrier oxidized by the echinoderm oxidase has a lower concentration (or is less active or accessible) in the unfertilized egg than in the fertilized.

Many authors have doubted the existence of cytochrome c in the egg, as it has never been possible to find the cytochrome bands (cf. Krahl and co-workers, 1941). Thus Korr (1939), who originally (1937) thought that cytochrome c was released at fertilization, and Ballentine (1940) suppose that some other link, situated nearer the substrate, is put into operation at fertilization. It is, however, not unlikely that cytochrome c may have a sufficient carrier capacity in the sea-urchin egg, and yet not have a concentration high enough to permit spectroscopic detection. It may be pointed out (a) that the sea-urchin egg has a comparatively low Q_{O_2} , and (b) that cytochrome c is far more catalytically effective when attached to the proper intracellular protein particles than when working in solution. It may also be that another carrier has the same function in respect to echinoderm oxidase as has cytochrome c to cytochrome oxidase. (Concerning the differences between echinoderm oxidase and cytochrome oxidase, cf. Borei, 1945, and Krahl and co-workers, 1941).

The surplus in respiration induced by fertilization is either merely an addition to the respiration of the unfertilized egg, or caused by the fact that the induced respira-

tion might compete with and depress the latter. The smooth increase in post-fertilization respiration from the level of the unfertilized egg in *Asterias* speaks in favor of the addition possibility (cf. Borei, 1948a and Borei and Lybing, 1949), provided the respiratory mechanisms in eggs of starfishes and sea-urchins may be freely compared. The facts concerning temperature characteristics of oxygen consumption rates revealed in *Arbacia*, do not in themselves distinguish between the two possibilities, nor do any facts gained in this investigation concerning *Psammechinus* material. Concerning the declining pre-fertilization respiration see, however, Borei (1949), where it is shown that this respiration part does not influence post-fertilization respiration.

Parallels have been drawn between the respiration of the unfertilized sea-urchin egg and the diapause egg of the grasshopper. In respect to temperature characteristics, the grasshopper diapause egg has a very low Q_{10} in comparison with that of the active stages (Bodine and Evans, 1932), which is in contrast to the state in the sea-urchin egg. Too much stress may thus not be laid on such comparisons.

SUMMARY

With Cartesian diver micro-respiration technique the temperature characteristics of the respiration of *Psammechinus miliaris* eggs were investigated:

1. Between 10 and 21° C. the gradual rise in oxygen consumption rate of the unfertilized egg is best represented by Krogh's standard curve. Q_{10} at 18° C. is around 2.5, at 10° C. around 3.5.
2. Fertilized eggs have the same temperature characteristics as unfertilized ones.
3. In respect to temperature characteristics just before and just after fertilization, two classes are distinguishable among the sea-urchins: The *Strongylocentrotus*-*Psammechinus* group with equal Q_{10} values before and after fertilization, and the *Arbacia* group with higher Q_{10} values before fertilization.
4. The significance of temperature characteristics for the biochemical processes involved in respiration of eggs before and after fertilization is discussed.

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INDEPENDENCE OF POST-FERTILIZATION RESPIRATION IN THE SEA-URCHIN EGG FROM THE LEVEL OF RESPIRA- TION BEFORE FERTILIZATION

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INTRODUCTION

The unfertilized sea-urchin egg just removed from the ovary respires at an oxygen consumption rate comparable with that of the newly fertilized egg (Borei, 1948a). After a few hours the respiration gradually drops, however, to a low, fairly constant level. This level represents the low respiration value of the unfertilized sea-urchin egg previously recorded in the literature. From this level there is a sudden jump at fertilization to the respiration rate of the newly fertilized egg. In *Psammechinus* the rate of the latter is usually three to four times that of the unfertilized egg at the constant level. For further details see Borei (1948a).

Now the question arises whether the increase in respiration after fertilization is merely an addition to the pre-fertilization respiration, or whether it competes with the existing respiration, eventually suppressing it completely.

In view of the concept (Runnström, 1930, Robbie, 1946) that the oxidase system in operation in the egg is the same before and after fertilization, and if parallels to events in the *Asterias* egg are considered (Borei and Lybing, 1949), the addition possibility is not unlikely. The difficulties arising in trying to interpret in this manner results from cyanide-inhibition experiments on fertilized eggs (Korr, 1937) could be avoided if Lindahl's (1940) findings are taken into account. He states that the cyanide-stable respiration arises under the influence of the inhibitor, and his view is strongly supported by Robbie's (1946) experiments.

On the other hand, Ballentine's (1940) concept that at fertilization a link in the dehydrogenase part of the oxidative chain is introduced, thus inducing an augmented respiration, offers a possibility that post-fertilization respiration, competing with that of pre-fertilization, may successfully suppress the latter, in spite of the fact (Robbie, 1946) that the same oxidase system is in operation both before and after fertilization. Korr, who first (1937) favored the view that cytochrome c is the link put into operation at fertilization, later (1939) abandons this and also turns to a concept that new ultimate substrate is released from precursors.

Runnström's (1930, 1932 and 1935) view that the oxidase is unsaturated with its substrate in the unfertilized egg, is equally consistent with both possibilities.

Borei and Lybing (1949) find that results from experiments on the temperature characteristics of sea-urchin eggs before and after fertilization can not decide between the two possibilities. It was stated by them that all facts at present known about sea-urchin egg respiration still leave the question open: It is just as possible to assume a simple addition as to believe in a competition.

It was thought that measurements of post-fertilization respiration in such experiments, where the eggs were fertilized at times corresponding to different points

on the decreasing respiration curve of the unfertilized egg, would help to elucidate the matter. If the rate of oxygen consumption before fertilization influenced the respiration of the fertilized egg, the addition possibility would have to be strongly considered. If not, one would be inclined to think that post-fertilization respiration is, as a whole, different from that before fertilization.

For a fuller discussion of previous literature on the biochemical aspects concerning sea-urchin egg respiration changes at fertilization, see Borei and Lybing (1949).

MATERIAL AND METHODS

The egg respiration of *Psammechinus miliaris* has recently (Borei, 1948a, Borei and Lybing, 1949) been studied in some detail. This species (phenotype S) was therefore chosen also in this investigation. For particulars concerning the material see Borei (1948a), Chapters 2.1, 3.111 and 3.21.

In order to make measurements possible on several lots of eggs taken from the same female and fertilized at subsequent times from the moment of removal from the ovary, one must use a method on the μ l. scale. Thus Cartesian diver micro-respiration technique was employed. Concerning technical points see Borei (1948a), Chapters 2.2 and 2.3. Diver charge Type I (Borei, 1948b) was used throughout.

All experiments were performed at 18° C. After completed diver measurements, the cells were taken out of the divers and observed as to condition and fertilizability. The only experiments accepted were those in which these controls turned out satisfactorily.

In the actual runs, the procedure was as follows: Eggs were removed from the ovary, a lot was immediately fertilized, then a diver was charged with unfertilized eggs and these were brought to measurement as rapidly as possible. (The first respiration values could thus be obtained ~20 mins. after egg removal from ovary.) This control diver was then continuously followed during all subsequent measurements on fertilized eggs from the same animal. From the first fertilized lot a diver was now charged. Subsequently new lots were fertilized and corresponding divers charged. In the experiments with fertilized eggs, the respiration rate at 120 minutes after fertilization, where the exponentially increasing respiration curve is still rather flat (see Borei, 1948a, Figure 3), was estimated and used for comparison. For unfertilized eggs a number of about 100, and for fertilized about 50, were found to be most suitable for charging the divers, which were of approximately 7 μ l. capacity.

RESULTS AND INTERPRETATIONS

The results from experiments on eggs from three females are represented in Figure 1.

It is obvious that respiration after fertilization reaches exactly the same level, irrespective of the height of the prevailing respiration at the moment of fertilization. It is of no importance whether the fertilization sets in at a very early moment, when the egg has just been removed from the ovary and accordingly respire at a very high rate, or whether it is effected a very long time after the removal, when the egg has already reached a fairly constant, low-rate respiration. In both cases the post-fertilization respiration will be the same.

In this connection it must be pointed out that the respiration of the fertilized egg in this investigation has been followed until more than nine hours after removal of the eggs from the ovary. This is considerably longer than in the cases reported by Borei (1948a), where the measurements were discontinued after about six hours. In no case, however, has there been observed any rise in the respiration rate at the end of the experiments, as might have been expected in consideration of the early findings of Warburg (1914) and Runnström (1928). Goldforb (referred to in Gerard and Rubenstein, 1934) found that the increase begins about five hours after shedding, and Tyler and Humason (1937) report a steady increase over the

whole measurement period. Runnström (1928) thinks that the increase is an indication of the "aging" of the egg (cf., however, Borei, 1948a, Chapter 3.113, who finds that over-ripe eggs have lower respiration than ripe ones and still lower than under-ripe ones). Tyler, Ricci and Horowitz (1938) found that the increase is avoided if the experiments are carried out under sterile conditions. It must be stated that all previous investigators have worked with Warburg technique. It might be that the dense packing of the eggs and the shaking conditions in this procedure support the growth of bacteria, whereas the Cartesian diver technique is more favorable in this respect. However, in this investigation no particular measures have been taken against bacterial contamination.

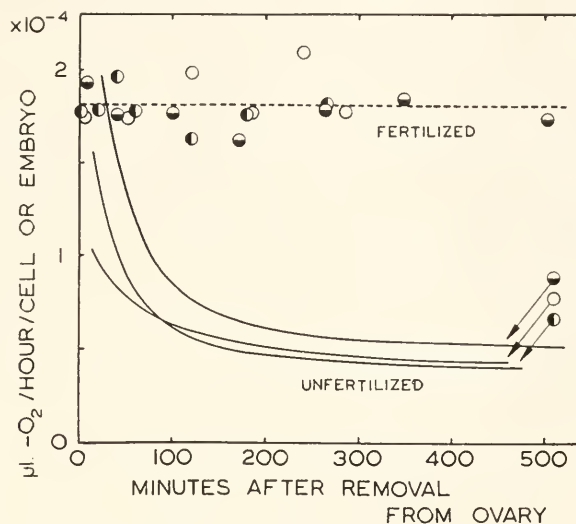


FIGURE 1. Oxygen consumption of unfertilized eggs and of fertilized eggs, fertilized at different times after the eggs' removal from the ovary. *Psammechinus miliaris*.

Each dot represents the respiration rate at 120 mins. after fertilization and is marked at the time of fertilization. $\circ$, $\ominus$ and $\bullet$ stand for the three females used. Temperature 18°C . Oxygen consumption rate of unfertilized eggs at 230 mins. after removal from ovary: $\circ = 0.50$, $\ominus = 0.58$ and $\bullet = 0.47 \times 10^{-4} \mu\text{l./cell}$ and hour (mean value found by Borei 1948a = 0.53); mean value of rates of fertilized eggs: $\circ = 1.85$, $\ominus = 1.78$ and $\bullet = 1.79 \times 10^{-4} \mu\text{l./embryo}$ and hour (Borei, 1948a = 1.84). For the respiration of fertilized eggs, the best fit according to the method of least squares is indicated by a dotted line.

Borei (1948a, Chapter 3.21) compares the quotient between respiration after fertilization and that before, and finds that the values from different sea-urchin species vary considerably, and moreover that for one and the same species, greatly differing values are reported. So for example for *Arbacia punctulata* quotients from 2.6 to 5.3 have been given. For *Psammechinus* the values 3.6 and 5.7 are recorded. On the other hand, if the conditions of measurement are well defined as to time from fertilization and from removal from ovary respectively, the quotient will become fairly constant, as the results of Borei (1948a, Chapters 3.112.2 and 3.21) show. Considering the possible influence of values of pre-fertilization respiration on the quotient, obtained at different points on the declining egg respiration curve, Borei (1948a) thinks that quotient values given in the literature are of minor importance for quantitative considerations concerning respiration changes at fertili-

zation, but merely show that the oxygen consumption of the unfertilized egg some few hours after its removal from the ovary is considerably lower than that of the fertilized egg some few hours after fertilization. The present investigation supports this concept thoroughly. If the quotients from the experiments in Figure 1 are recorded (Table I), it is clearly seen that the quotient value will become smaller and smaller the closer the time of fertilization lies to that of egg removal. It would rather seem that values < 1.0 could be obtained in the earliest experiments, i.e. that the respiration of the just removed egg is actually higher than that of the fertilized egg during the first hours of development (cf. Borei, 1948a).

TABLE I

Quotients between oxygen consumption rate 120 mins. after fertilization and oxygen consumption rate of unfertilized eggs at the moment of fertilization. Psammechinus miliaris

Same experiments and denotations as in Figure 1. Value given by Borei (1948a) for 230 mins. after egg removal from ovary = 3.6. In quotients marked * respiration values of unfertilized eggs are obtained graphically from extrapolated curves.

○		◐		◑	
Mins. after egg removal	Quotient	Mins. after egg removal	Quotient	Mins. after egg removal	Quotient
6	$< 1.6^*$	8	$< 1.0^*$	2	$< 1.0^*$
52	2.3	40	1.2	21	1.2
120	3.3	100	2.2	41	2.5
185	3.5	170	2.5	60	2.8
241	4.3	264	3.2	120	3.1
286	3.8	349	3.5	179	3.6
		502	3.4	265	3.9

It would appear from the presented data, as the value of the pre-fertilization respiration rate seems to be of no importance for the oxygen consumption rate after fertilization, either that pre-fertilization respiration constitutes no integral part of the respiration after fertilization, or that fertilization brings about a release from inhibiting factors active on respiration in the unfertilized egg. As the oxidase system is probably the same both before and after fertilization (Runnström, 1930, Robbie, 1946; see also Introduction of the present paper), it is reasonable to suppose that changes occur at fertilization in those parts of the system which are situated between the oxidase and the dehydrogenases. The dehydrogenases themselves, however, are not likely to be affected. (Dimethyl-p-phenylenediamine experiments by Runnström, 1930 and 1932, Örström, 1932, Borei and Renvall, 1949; hydroquinone, Runnström, 1930; pyocyanine, Runnström, 1935; methylene blue, Runnström, 1930; cf., however, Ballentine, 1940, who claims that the dehydrogenases are not capable of maximum activity in the unfertilized egg.) (Cf. Korr, 1939: "release of substrate from precursors.")

From experiments on the respiratory quotient of pre- and post-fertilization respiration in the sea-urchin egg, it appears very probable that different substrates are utilized before and after fertilization. These changes in RQ upon fertilization,

which support the view that post-fertilization respiration competes with that of pre-fertilization, eventually suppressing it more or less completely, are seen from Table II.

It must, however, be kept in mind that a constant respiration part is assumed for the unfertilized egg (Borei, 1948a, Chapter 3.112.1) as well as a decreasing part. The above-presented data do not indicate whether even the constant respiration part is abolished at fertilization. It is still an open question whether this part of pre-fertilization respiration survives fertilization or not.

TABLE II

RQ of sea-urchin egg respiration before and after fertilization

Time in relation to fertilization	RQ	Species	Author
Before*	1-1.2	<i>Psammechinus</i>	Borei (1934)
0-30 mins. after	0.84		Laser and Rothschild (1939)
30-40 mins. after	0.78		Borei (1934)
40-50 mins. after	0.64		
35-50 mins. after	0.66		Laser and Rothschild (1939)
1-2 hr. after	0.73	<i>Paracentrotus</i>	Öhman (1940)
7-8 hr. after	0.85		Ephrussi (1933)
2-cell stage-hatching	0.8		

* Ashbel (1930) finds the value 1.06 before fertilization (Arbacia).

SUMMARY

Using Cartesian diver micro-respiration technique, it was found that in *Psammechinus miliaris* the rate of respiration of the newly fertilized egg is independent of the rate of respiration of the unfertilized egg at the moment of fertilization.

The quotient (respiration after fertilization)/(respiration before fertilization) was found to decrease considerably (probably even to values < 1.0) if the time interval between egg removal from the ovary and fertilization was diminished. If the decreasing part of pre-fertilization respiration is given time to disappear before fertilization, the quotient lies between 3 and 4.

It is thought possible that the decreasing respiration part of the unfertilized egg is abolished upon fertilization, due to probable changes in the function of members of the oxidizing system, situated between the oxidase and the dehydrogenases. It cannot be decided from the experiments whether the constant respiration part of the unfertilized egg still participates in the respiration of the fertilized egg or not.

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STUDIES ON THE THERMAL DEATH OF *HYALELLA AZTECA* SAUSSURE¹

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INTRODUCTION

Much work has been done in the past half century concerning the effects of temperature on the survival of organisms. However, little has been done in that respect with the Crustacea, particularly the fresh water forms.

A study was made from June 16, 1947, to August 15, 1947, concerning the effects of temperature on the survival and death rates of the amphipod *Hyaella azteca* Saussure (*Hyaella knickerbockeri* Bate, *Hyaella dentata* Smith). This amphipod is abundant in the waters of Little Miller's Bay in West Okoboji Lake, Dickinson County, Iowa, at the site of the Iowa Lakeside Laboratory, where the investigation was conducted.

HISTORICAL SURVEY

Geisler (1944) suggests that rate of development in *Hyaella azteca* is directly related to the temperature, but does not record the effects of higher temperatures. For marine copepods and decapods, Huntsman and Sparks (1924) report heat death at temperatures between 22° and 33° C. when the animals were exposed to temperatures rising at the average rate of 0.2° C. per minute. Brown (1928) reported a temperature characteristic, μ , of 187,000 calories from 35° to 41° C. for thermal death in *Daphnia magna*.

MATERIALS AND METHODS

Numerous *Hyaella* were secured daily by placing fresh masses of the green alga, *Cladophora fracta*, in which they feed, in a pail of lake water. Amphipods which came to the surface of the water were transferred to a stock tank of fresh lake water at 20° to 22° C. by means of a tea strainer. This lake water was partially changed daily and replaced weekly, and was used in all experiments. The temperature of the tank water was about the same as that of the natural water where the animals were taken.

The water baths used in the experiments were five-gallon containers heated electrically and controlled manually to $\pm 0.2^\circ$ C.

¹ Undertaken in partial fulfillment of the degree of Master of Science at the State University of Iowa. The author wishes to express his gratitude for helpful criticism of the initial and final drafts of the paper on the part of Dr. Theodore Louis Jahn of the University of California, Los Angeles, formerly of the University of Iowa, and Dr. Robert L. King of the University of Iowa, at whose suggestion the problem was begun.

² On leave of absence for research and study at the University of California, Los Angeles.

In short exposures several hundred *Hyaella* were dipped from the stock tank with a tea strainer, over which a muslin square was then fastened and held taut with a rubber band. The strainer was immersed and oscillated in the water bath for the desired period. Then the *Hyaella* and the strainer were transferred quickly to enamelled basins of fresh lake water at 20° to 22° C.

In exposures longer than thirty seconds small tin cans with tops and bottoms removed were employed. Muslin squares fastened over the open ends kept the amphipods confined, but exposed to the water. A number of these tins were placed in the water bath and removed as desired.

The water bath was stirred between short exposures, and during exposures longer than thirty seconds was continually aerated with compressed air. The tins containing the *Hyaella* were placed on a wire platform as near the center of the water bath as possible, and at least six inches away from the source of heat.

Exposed animals were allowed to remain in recovery basins four or more hours before counting. Counting was usually done six to eight hours after exposure. Check counts showed that injured animals which were alive four hours after exposure were always dead after sixteen hours. Therefore, injured animals were counted as dead. Control animals remained alive, except for rare exceptions, after twenty-four hours in the basins.

Water from each basin was individually strained through muslin to collect the the amphipods for counting. The muslin was spread flat on a moist, concrete slab for counting under a sixty-watt electric bulb. Because of negative tropism to light (Phipps, 1915), the living amphipods crawled toward the periphery. A few cubic centimeters of water dropped at the center of the muslin hastened the outward movement. After thus separating the living and dead, the living were counted first. Those evidencing injury or feebleness when stimulated with water or the points of a pair of tweezers were counted as dead.

Mature specimens were separated into two groups; so also were immature specimens. Those longer than 7 mm. were considered very large adults; those from 3½ to 7 mm. as large adults; those from 2½ to 3 mm. as medium-sized adolescents; and those still less as small and juvenile (Geisler, 1944). A separate count was taken according to sexes for each of the adult groups on the basis of salient characteristics (Geisler, 1944) as seen through a dissecting scope.

Time intervals were determined with a watch calibrated in fifths of a second.

EXPERIMENTAL RESULTS

Survival in constant temperature baths

Constant temperatures used included one degree intervals C. from 38° to 50°. Tests were also run at 36.5°, 35° and 33°.

Thermal death times and temperatures here shown are those at which fifty percent of the organisms survived.

At 50°, less than one second was sufficient time to cause thermal death. At lower temperatures the time increased gradually so that at 40° an exposure of 75 seconds was needed. Below 40° the increase in time required to kill was very sharp, so that at 33° more than eleven hours (39,600 seconds) was necessary (Table 1).

Size and survival in constant temperature baths

Age and size are directly correlated in *Hyalella*; the larger the animal, the older it is (Geisler, 1944). Large adults showed the greatest resistance, usually higher than the average figure for the total of all simultaneously exposed. Very large adults varied in their resistance, but their rate of survival usually approached the average. Medium adolescents showed still less resistance; and small juveniles were least resistant (Table 1).

TABLE 1

Heat death for Hyalella azteca in constant temperature baths

Degrees C.	Time exposed in seconds	Per cent survivors					Total number animals exposed
		Total animals exposed	Very large adults	Large adults	Medium adolescents	Small juveniles	
50.0	1	50.8	52.1	62.5	45.6	43.3	240
49.0	4	51.2	64.8	59.4	47.5	44.4	416
48.0	7	50.3	48.7	51.8	52.0	47.6	320
47.0	8	50.8	58.3	57.2	48.2	46.6	114
46.0	12	49.3	31.7	51.9	54.3	47.1	162
45.0	14	47.1	52.2	65.4	36.3	43.6	318
44.0	15	55.0	40.1	56.2	56.7	49.4	623
43.0	24	49.5	58.5	63.6	43.0	43.3	656
42.0	39	49.9	52.8	56.4	49.1	37.9	204
41.0	60	47.6	33.6	67.6	51.3	46.1	432
40.0	75	49.6	49.9	68.8	47.6	46.8	626
39.0	135	49.3	49.3	58.4	44.6	48.4	636
38.0	240	48.9	49.2	55.8	45.0	46.9	547
36.5	1,800	49.9	44.8	52.9	51.1	53.6	210
35.0	6,300	51.8	50.2	56.2	46.1	46.8	583
33.0	39,600	53.7	51.2	51.9	51.5	48.2	146

Sex and survival in constant temperature baths

No valid evidence was found to indicate that sex affects resistance to heat in constant temperature baths. For all adult specimens, male survivors outnumbered females in sixteen out of thirty-two cases.

Survival in rising temperature baths

Using the same equipment as that employed for the constant temperature baths, *Hyalella* were exposed to four average rates of temperature rise, beginning at 20° to 22° C. Rates of rise were: 0.375° per minute; 0.261° per minute; 0.150° per minute; and 0.036° per minute. At lower temperatures the rate of rise per degree C. was more rapid than at higher temperatures. The rates of rise are here expressed as average rates, in order to make them comparable to those of other investigators who previously encountered the same difficulty (Huntsman and Sparks, 1924). Within the rates of rise investigated, thermal death did not occur below 39° C. and always was found above 41° C. (Table 2).

TABLE 2

Heat death in Hyulella azteca in rising temperature baths

Rate* of rise in degrees C. per minute from 20° to 22° as a base	Degrees C.	Total animals exposed	Per cent survivors				
			Total animals	Very large adults	Large adults	Medium adolescents	Small juveniles
0.375	37.0	178	89.4	94.6	92.6	88.8	74.1
	38.0	212	84.4	85.7	77.8	75.8	75.0
	39.0	189	87.8	93.7	88.8	82.7	78.3
	40.0	201	51.7	52.7	50.2	50.0	51.8
	41.0	170	60.2	69.7	54.8	52.6	50.0
	42.0 to 45.0 }	#200@					
0.261	37.0	462	85.9	95.5	96.3	89.1	83.1
	38.0	470	81.1	91.1	83.7	77.2	76.7
	39.0	456	83.9	93.1	83.1	83.7	78.6
	40.0	534	68.1	81.0	79.9	63.1	64.6
	41.0	340	27.6	31.2	23.6	26.5	29.0
	42.0 to 45.0 }	#400@					
0.150	37.0	421	93.5	96.4	95.8	86.5	87.5
	38.0	492	82.9	92.3	85.8	76.8	77.1
	39.0	493	77.4	88.6	76.9	75.4	66.6
	40.0	430	70.7	75.0	73.0	65.3	65.5
	41.0 to 45.0 }	#400@					
0.036	36.0	464	94.8	97.6	96.0	89.6	91.8
	37.0	306	86.9	94.6	95.6	82.5	78.7
	38.0	493	65.9	73.5	81.5	59.8	55.9
	39.0	194	29.3	12.7	39.0	42.0	25.6
	40.0 to 45.0 }	#350@					

* Average rate of rise.

Approximate number at each degree of temperature within the bracketed limits; no accurate count taken since all were dead.

Size and resistance to rising temperatures

Very large adults demonstrated the highest resistance in rising temperature baths, except at the slowest rate of rise. Large adults, medium adolescents, and small juveniles showed, respectively, less resistance (Table 2).

Sex and resistance to rising temperatures

No evidence was found that sex causes any variance in resistance to rising tem-

peratures. Male adult survivors in some trials outnumbered females, and vice versa, but never in significant numbers.

Adjustment to rising temperatures of water

A temporary adjustment to rising temperatures was noted. For example, organisms plunged into and continuously exposed to a pre-heated constant temperature bath at 38° C. reached thermal death in four minutes. Within the rates of rise investigated, thermal death was not found to occur at 38°, 87.8 per cent still surviving at that temperature at the fastest rate of rise, and 65.9 per cent surviving at the slowest rate of rise (Table 2).

Temperature coefficients for thermal death

Adaptations of the v'ant Hoff-Arrhenius equation are often used to express the rate of progress in biological reactions, although it is possible that such characteristics are more descriptive than analytically accurate.

Computation and comparison of Q_{10} for a number of temperature ranges within the full range investigated revealed that although death occurs more quickly at higher temperatures, the rate at which the lethal effect progresses decreases as the temperature increases. The decrease in Q_{10} was very marked for intervals below 40° C. Above that temperature there was a sharp break in the deceleration of the rate of progress and the Q_{10} variance was not so great (Table 3).

TABLE 3

*Temperature characteristics for Hyalella azteca for
thermal death in constant temperature baths*

Temperature intervals in degrees C.	Q_{10}
33.0-35.0	9,768.00
35.0-40.0	7,056.00
38.0-43.0	100.00
40.0-45.0	28.91
43.0-48.0	11.75
45.0-50.0	196.00*

* Apparent divergence may be due to experimental inaccuracies.

SUMMARY

1. Thermal death occurs in *Hyalella azteca* at constant temperatures from 33° to 50° C. The time required to produce thermal death varies from more than eleven hours (39,600 seconds) at 33°, to less than one second at 50° C.

2. Comparison of Q_{10} values for narrow ranges within the broad range of temperatures investigated indicates a marked decrease of Q_{10} values at higher temperatures in spite of a more rapid lethal effect.

3. Thermal death occurred in rising temperatures, the slower the rate of rise, the lower the killing temperature, being not below 39° nor above 41° for the rates of rise investigated.

4. A temporary adjustment was found to occur to rising temperatures, delaying thermal death at a given temperature for some time past the period necessary to kill on immersion in the constant temperature bath at the given temperature.

5. Resistance to the effects of heat appears to be directly related to the size and age of the animal, the older and larger the animal the greater the resistance, except for the largest animals (which might have reached a state of senility).

6. Resistance to the effects of heat does not appear to be related to sex in *Hyaella azteca*.

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REGENERATION IN AN EARTHWORM, *EISENIA FOETIDA* (SAVIGNY) 1826. I. ANTERIOR REGENERATION

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These contributions present the results of an attempt to obtain for one particular species of earthworm complete characterization of regenerative capacity with reference to exact levels. In this part anterior regeneration, by posterior substrates only, is considered.

SUMMARY OF PREVIOUS WORK

Information available as to the morphological nature and segmental constitution of anterior regenerates on posterior substrates is summarized, with certain reservations, in Table I.

In earlier work on *E. foetida*, as well as other species of earthworms, determination of morphological nature of regenerates seemed unnecessary. Later, Michel (1898, p. 283), recalling Bonnet's heteromorphic tails in aquatic Oligochaeta, suggested that two of his own anterior regenerates, as well as some of those of Joest and Rievel, were caudal. Although anterior heteromorphosis was definitely confirmed by Morgan (1899) no attempt was made then or since to clarify the situation, and in particular to determine the limits of homomorphic head and heteromorphic tail regeneration.

The consequent uncertainty as to morphological nature of regenerates at a considerable number of levels and even as to the levels (because of postregeneration estimation), as well as absence of data for numbers of levels and paucity at other levels, indicated the advisability of a systematic investigation of regeneration at each level from $\frac{1}{2}$ posteriorly.

MATERIALS AND METHODS

Material was first secured from a heap of decaying leaves, later from manure heaps. Worms were kept in moist filter paper or paper towelling until the gut was cleared. Individuals with any indication of damage by collecting, disease, abnormality, homoeosis or previous regeneration were rigorously rejected, and only those which were clitellate, or which had been clitellate when brought into the laboratory, were used. Animals were kept throughout at ordinary room temperature, which in winter probably was never above 68° F.

Anaesthesia was brought about in 0.2 per cent chloretone. Transections were made under a dissecting binocular microscope exactly across the animal on an inter-segmental furrow.

After operation worms were placed in water until recovery from anaesthetic and were then transferred to filter paper, paper towelling, or cheesecloth. On several

TABLE I
Anterior regeneration in Eisenia foetida

Level	Regenerate										Comments	Author	Date	Page
	Cephalic							* Uncertain	Caudal	None				
	Number of segments													
	2	3	4	5	6	7	?							
1/2											See note (a)			
2/3	6											Morgan	1895	447
3/4	5 4	9 1										Morgan Michel	1895 1898	447 261
4/5	1 3	8 1 10	5 1									Morgan Hescheler Michel	1895 1896 1898	448 228 262
5/6	1	2 8	3 5								See note (b)	Morgan Hescheler Michel	1895 1896 1898	448 228-31 268
EL		5	5	1							See notes (c) (d)	Morgan	1895	453
6/7 EL	1	2 4	3 6	1								Hescheler Morgan	1896 1895	228 453-6
7/8 EL		1 1				1					See note (e)	Hescheler Michel Morgan	1896 1898 1895	228 262 453
8/9 EL	1	3	1 5	3 1	1							Michel Morgan	1898 1895	262 455
9/10 EL	1	1		3								Morgan	1895	453-6
10/11 EL			3 2	5	3 1			1			U = "Imp"	Morgan Carpenter Morgan	1895 1948 1895	451 625-6 455
11/12 EL			3 1	1 1				1			U = "2 (or three very imper- fect)"	Michel Morgan	1898 1895	263 456
12/13 EL	1	1 1	2 1	1				1 2	0 0	0	U:S=indistinct 100-0-0% _c	Morgan Michel Dimon Morgan	1895 1898 1904 1895	451-6 263 350 456
13/14 EL		1										Morgan	1895	456
14/15				1				10	0	0	100-0-0% _o	Morgan Dimon	1895 1904	451 350

TABLE I—*Continued*

Level	Regenerate										Comments	Author	Date	Page
	Cephalic							Uncertain *	Caudal	None				
	Number of segments													
	2	3	4	5	6	7	?							
15/16 EL						22 3	2# 4	0 1?	5	92-8-0% ? = "possibly a new tail"	Dimon Morgan	1904 1902	350 579	
16/17 EL						11	1# 2	0		92-8-0% 3-4 S, "not regenerated (mouth present)" (See note (f))	Dimon Morgan	1904 1895	350 455	
17/18						12	1#	0		92-8-0%	Dimon	1904	350	
18/19						26	5#	2		79-15-6%	Dimon	1904	350	
19/20						3	1 2#	? ?		U = "4 or 5 S" See note (g)	Morgan Dimon	1895 1904	451 350	
20/21 EL									1	17 S See note (h)	Morgan	1899	409	
?						4	7#	12		17-30.5-52.5% See note (g)	Dimon	1904	350	
22/23						2				Very imperfect	Morgan	1895	452	
23/24 EL						1 1				Very imperfect 3 or 4 S, imperfect	Morgan Morgan	1895 1895	452 456	
24/25						2				See note (h)	Morgan	1897	574	
EL 25/26						1		1		T = 17 S See notes (h) and (i)	Morgan	1899	40	
EL 30/31						1		1		H = 7 or 8 S See note (h) T = 21 S	Morgan	1899	408	
EL 34/35			1								Morgan	1901	7, Fig. G	
EL 35/36								1		(L 63 S) T = 15 + S See notes (h) and (i)	Morgan	1895	455	
L 75 S								1		35 S	Morgan	1899	409	
EL 50/51			1			2	14	15		T = 5-25 S	Morgan Morgan	1901 1902	7, Fig. H 579	

TABLE I—*Continued*

Level	Regenerate										Comments	Author	Date	Page
	Cephalic							* Uncertain	Caudal	None				
	Number of segments													
	2	3	4	5	6	7	?							
L20S										5		Michel	1898	263
L10S										5		Michel	1898	263
L5S										5		Michel	1898	263
L12-7S										98		Morgan	1897	575-6

NOTES TO TABLE I

(a) "Attempts made to cut off 1 and 2 segments" (Morgan, 1895, p. 449). As a result of confusion re numbering of containers there was but one case in which it was thought "one segment must have been cut off" and that specimen could have been a posterior homoeotic.

(b) The last two specimens in Morgan's Table IV were homoeotic and are here excluded. The three specimens next above are assumed to have been normal. (Homoeotics are excluded here, as well as from author's operations, to obviate possibility of complications resulting from a previous regeneration and because gradients cannot be expected to be the same as in normal specimens.)

(c) Amputations, apparently as a result of operating without anaesthesia, were often diagonal (Morgan, 1895, p. 457, also Korschelt), or if transverse then at an intra- rather than intersegmental level. In each case a portion of a segment is treated as if a whole segment, i.e. if $10\frac{1}{4}$ or $10\frac{1}{2}$ segments were removed from the anterior end, the level of regeneration is still considered to be 10/11. In favor of this convention is Morgan's conclusion, after study of deliberately made very diagonal cuts, that simultaneous completion of missing parts of segments did not interfere with replacement of those metameres that had been completely amputated (1895, p. 457).

(d) EL estimated level. Level of amputation in many operations was estimated *after* regeneration and from one of the following landmarks (Morgan, 1895, pp. 450 and 452): (1) Position of vasa deferentia, i.e. location of male pores. Subject to variation of six segmental levels (Morgan, 1895, p. 403). (2) Location of seminal receptacles, apparently thought to be three pairs. *Eisenia foetida* has only two pairs of spermathecae but four pairs of seminal vesicles. These landmarks are doubtful. [If three pairs of spermathecae were present another species was involved, possibly *Dendrobaena octaedra* (Savigny) 1826 or *Allolobophora chlorotica* (Savigny) 1826, both of which are found in compost heaps and apparently have been confused with *foetida*.] (3) Location of clitellum. That may begin on any of segments xxiv-xxvii and end on xxxi-xxxiv, a variation of three to four levels. Pre-clitellar amputation was variously listed as at 20/21, 25/26, and postclitellar as at 25/26, 30/31, 35/36. (4) and (5) The middle and the end of the body, the former regarded as at 50/51 and the latter as the hundredth segment. Number of segments varies from 67-125.

Actual variations, when recognizable, were: for (3) of seven segmental levels, i.e. 19/20-26/27 and 31/32-38/39, for (4) and (5) to about 20 levels, 41/42-64/65, etc.

Postregeneration determination of level of amputation would probably render unlikely detection of reorganization of substrate segments. Such reorganization, in some species, could affect the determination by one to three segmental levels.

In certain of Morgan's cases it is not clear whether levels mentioned were determined or estimated.

(e) Number of segments of other regenerates at this level "très variable."

(f) The regenerate segments were "very irregular." The characterization "not regenerated

occasions when the supply of cloth had been exhausted, worms were kept in large crystallizing dishes in water just sufficient to cover the bottom and keep the animals moist. Although *E. foetida* appeared to do as well in water, in cool weather, as in moist cloth, the method is not recommended, for in later work several long series of operated animals were completely lost over night. To prevent accumulation of metabolic wastes, water or paper was changed (or cloth washed) daily, except on Sunday when the museum was closed.

Specimens were killed so as to insure uniform contraction and were then preserved in formalin.

The experiments were carried out mainly during a sabbatical leave in the States in 1926-27. Shortly before Japan entered the war, a summary of the results obtained was prepared and sent home from Burma. Original records, as well as specimens, were lost in the sack of Rangoon.

The author's thanks are extended to Prof. G. H. Parker for provision of laboratory facilities at Harvard University during the academic year 1926-27, to Prof. H. W. Rand for similar facilities in the U. S. Fish Commission building at Woods Hole during the summer of 1927, to Dr. Esther Carpenter for care of operated animals while the author was ill.

NOMENCLATURE

In one and the same article, an author once used "posterior end" to refer to: a posterior regenerate regardless of size; an anterior regenerate (heteromorphic); the anal region of an adult worm; and long posterior portions of varying lengths up to a half or more of adult size. Similarly "anterior end" has had various meanings, including even that of tail (heteromorphic). Most confusing, however, has been a failure to distinguish adequately in discussions between the regeneration taking place at a single surface of amputation and that taking place at exactly the same level when there are two cut surfaces. In an attempt to avoid further complications, an effort has been made to restrict terms and phrases consistently to the meanings given herewith.

(mouth present)" may refer to an anally sculptured cicatrix. Such sculpturing may be preliminary to growth of a tail regenerate.

(g) Results of all operations behind 18/19 were lumped together. Mention was, however, made of three "B" heads at 19/20 which have also been listed above at that level.

(h) No data as to number of segments in 1897 regenerates (Morgan, pp. 573-574) and no clues to warrant guesses as to nature of regenerates.

(i) Results of 30 operations (Morgan, 1902, pp. 578-579) omitted because of uncertainty: (1) as to level of operation, said to have been "just behind the girdle (about the 25th segment)," i.e. either at 25/26 or 34/35; (2) as to nature of substrate, i.e. whether posterior or a two-surfaced fragment. Three months after operation, four specimens having died, the container had 36 specimens which were not examined for autotomy. *Nevertheless*, presence of one distinct new head and 14 doubtful regenerates, of which "probably more were heads than tails" may be of considerable importance.

Presence of extra worms in containers may have another explanation than autotomy. Just hatched juveniles are exceedingly difficult to find in either manure or soil. In absence of sterilization of the manure used for culture medium, there was time, during the months allowed for regeneration, for young to attain adult size. In this connection a belief that regenerates became indistinguishable from substrates is perhaps important (Morgan, 1895, p. 424).

Regenerates referred to this class by Dimon were not characterized in any way. * Some "doubtful" regenerates of other authors are also included here. Others, that appear also to be doubtful, have been included in part.

In place of regenerant and regenerate, which are easily confused, *substrate* and *regenerate* are used respectively, to designate the portion of the original worm on which the new growth is formed and the new growth thus formed at the cut surface. This is in continuation of previous practice (Gates, 1941).

Posterior substrate refers to any posterior portion of the body, regardless of size, extending forward from the anal region to a single anterior cut surface.

Healing after amputation may be *cicatricial* or *enteroparietal*. In the first, a cicatrix is formed across the cut surface, while in the second, cut edges of gut and body wall apparently heal together without definitely recognizable cicatricial tissue.

A regenerate with no externally recognizable differentiation is a *bud* (indeterminate). As indications of buccal or anal sculpturing become recognizable, further characterization as cephalic or caudal is possible. With appearance of metameric differentiation the regenerate is a head or a tail.

A considerable degree of deviation from normal structure may be possible in a regenerate without affecting its caudal or cephalic nature. Such variant regenerates are abnormal. A regenerate without cephalic or caudal characteristics, or with a mixture of cephalic and caudal characteristics, or with bifurcations, is a *monstrosity*. A growth without indication of caudal or cephalic nature is an indeterminate monstrosity.

A metamERICALLY normal regenerate may be *equimeric*, *hypermeric*, or *hypomeric*, depending on whether it has the same number of segments, more than, or fewer than the excised portion.

Heteromorphosis indicates a more or less normal structure in a reversed or abnormal direction. A head at a posterior amputation and a tail at an anterior amputation is heteromorphic. Homomorphic distinguishes the head or tail in normal position or direction.

Levels are designated in two ways, by reference to the segments, as xxvi, and to the furrows bounding the segment as 25/26 and 26/27. The Roman numeral in lower case means the twenty-sixth segment beginning with the buccal as i; the prostomium of the Oligochaeta is not counted as a segment. The fractions refer to the intersegmental furrow at the anterior and posterior margin of segment xxvi, and make possible, with shorthand brevity, exact designation of level. Indication of level of amputation merely by reference to the segment, as "at the twenty-sixth segment," may be inadequate unless the context indicates which of the two possible levels, anterior or posterior, is involved. EL before the fraction means *estimated level*, the estimate usually that of the original author, otherwise made in accordance with his custom so far as is possible.

The anal region of the body forward to the first complete intersegmental furrow is not regarded as an ordinary metamere but for purposes of segmental enumeration is taken as one segment (see Gates, 1948). Posterior substrates of unknown location with reference to the antero-posterior axis are characterized by a designation such as L14S, in that case meaning the last fourteen segments.

Homocosis, as ordinarily used in connection with earthworms, means: presence of an organ or pair of organs, or a series of organs, in a segment or series of segments, other than that, or those, in which usually or normally found. It refers primarily to individual variation within a species; secondarily, to phylogenetic variation, for a species or a genus may be homoeotic with reference to other species in the genus, or other genera in the family. In case of individual homocosis, the dislocation may involve one or both organs of a pair in a segment. The former is asymmetrical homocosis, the latter symmetrical.

SUMMARY OF RESULTS

Healing at cuts in an anterior portion of the body was cicatricial, the cicatrix a low, flat-surfaced, circular disc without recognizable sculpturing. In some specimens no further development was recognizable. In others the cicatricial disc gradually was protruded as a small, rather conical bud at first apparently unmarked by any sculpturing. In several cases the growth of the bud was inhibited at that stage. In the remainder the distal portion became sculptured to indicate a prostomium and mouth. Intersegmental furrows, setae, and finally pigment usually became recognizable in that order.

Regenerates always remained distinguishable from substrates by differences in pigmentation, segment size, setal intervals, etc.

Several months' starvation resulted in reduction of size of substrates but no macroscopically recognizable reorganization was noted, either externally or internally, behind the level of amputation.

Reproductive organs were not found, in regenerate or (as result of reorganization) in substrate.

A. After a single cut

All substrates with cut surfaces at levels from 8/9 anteriorly, with one exception, regenerated. At each level behind 8/9 one or more of the substrates did not survive operation long enough to regenerate or else failed to regenerate if surviving. Highest percentages of failure to regenerate were in the region around 25/26. Further posteriorly, survival was better and percentages of successful regeneration higher. Results just mentioned were, however, minimal, as inhibited buds, rare monstrosities (indeterminate) and certain conditions to be considered later on were recorded as failures (to produce a more or less normal head or tail).

Head regenerates were obtained at levels 1/2–23/24 only. Equimeric heads were obtained at levels 1/2–8/9 inclusive. Three regenerates at 4/5 were hypermeric (+ 1). All head regenerates at levels 9/10–23/24 were hypomeric, the maximum number of segments obtained being six. In a later series of operations, E43, of three head regenerates at 8/9, one had five, another had six, and a third had nine and a half segments, the half segment wedge-shaped and on the right side (+ 1½).

Heteromorphic tail regenerates were obtained, once each at levels 20/21 and 23/24, and from 24/25 to 54/55. The largest number of setigerous segments differentiated in such heteromorphic tails was 25 at 40/41, the evidence available indicating increase in number of segments posteriorly to 40/41 and then a decrease.

At levels behind 54/55 no regeneration whatever, including even buds and monstrosities, was obtained though numbers of substrates were under observation three to four months.

B. After a previous regeneration

In attempts to test for the effects of previous regeneration on anterior regeneration, several series of operations were made of which the following are mentioned.

In series E41 posterior portions were removed at 34/35 and 35/36 and the substrates (anterior) were allowed to regenerate for twenty-three days. At that time the anterior eight segments were removed and discarded. Of the surviving substrates (8/9–34/35 or 35/36 + a tail regenerate), four regenerated heads anteriorly. Three were hypomeric with six segments each, and one was hypermeric with nine segments (+ 1).

In series E58 the last ten segments were removed from specimens having one hundred or more segments. At the end of twenty-two days' regeneration, anterior portions were removed so as to leave ten or fifteen segments of the original substrates along with the tail regenerates. One of these small substrates had already produced a bud at the anterior cut surface by the seventh day, at which time circumstances compelled termination of the experiment. Anterior regeneration in

this series, would, if completed, have taken place at levels behind 75/76, while normal posterior substrates, unconditioned by a previous regeneration, failed to regenerate at levels behind 54/55.

In series E49 the posterior portion of the body was removed at 70/71. The anterior substrates were allowed to regenerate posteriorly for eighty days. At that time the tail regenerates were removed at the level of regeneration. One such tail regenerate, then acting as substrate, produced in twenty-seven days, at the anterior cut surface (level 70/71 with reference to location on axis of original worm), a heteromorphic tail, unpigmented but with six setigerous segments and a small anal region without indication of production of further segments. Final substrates in this series were 7 to 10 mm. long and of 30–41 setigerous segments.

C. After starvation

To test for the effect of starvation, the following experiment was run (see also series E49 above for regeneration after 80 days' starvation). From worms that had been starved for seventy days or longer, the anterior five or six segments were removed. Each surviving substrate regenerated a hypomeric head (–1 to –3) with metameric differentiation complete and normal.

DISCUSSION

A first step towards obtaining a complete characterization of regenerative capacity in *E. foetida* is determination of the morphological nature of the regenerate produced anteriorly, at each intersegmental level along the axis, by posterior substrates, as well as the number of segments in such regenerates. The latter, often neglected in the past apparently as of little importance, is of some interest with regard to morphogenesis in the Lumbricidae.

Hypermermy in head regenerates has now been recorded for the first time in *E. foetida*, and at two different levels, one of which, 8/9, is fairly well back. Hescheler (1896, p. 93) once secured a regenerate with more segments than had been removed but in a series of successive regenerations by a single individual, the worm even then still hypomeric by two segments (removal of $6\frac{1}{2}$ segments, regeneration of $5\frac{1}{2}$; removal of 4, regeneration of 2; removal of 2, regeneration of 3). One hitherto unnoticed case of hypermermy in the Lumbricidae has been found—regeneration of four segments after removal of three by a specimen from which the nerve cord had been removed from the next two metameres behind the level of amputation (species unidentified, Goldfarb, 1909, p. 703, Table 4, No. 1.41).

Hypermeric regenerates are of especial interest in connection with the problem of the origin of posterior homoeosis. In *E. foetida* posterior homoeosis of one segment only has been recorded and now in regenerates hypermermy of one segment only. As all cases of symmetrical homoeosis in the species can now be considered to have resulted from hypomeric or hypermeric regeneration, postulation of some unknown embryonic cause is no longer necessary.

The new data as to segment number in homomorphic anterior regenerates provides confirmation of the cephalic nature of Michel's and Hescheler's regenerates of seven segments and of Morgan's regenerate of "7 or 8" segments, all of which seem to have been overlooked hitherto.

Presence in a head regenerate of nine segments may indicate a possibility of equimeric regeneration back to 9/10 but is of especial interest in connection with the problem of the constitution of the "head." In the Oligochaeta homomorphic anterior regeneration is generally thought to be restricted to replacement of the "head." The latter, in the Lumbricidae, has been thought to comprise five segments only. Six, seven, and eight (?) -segment head regenerates obtained by Michel and Morgan in *E. foetida* (Table I), and a six-segment regenerate at 9/10, as well as a seven-segment regenerate at 12/13 in *Allolobophora terrestris* (Hescheler, 1896), should have been taken into consideration in this connection. Carpenter's (1948) regenerates of six segments (Table I), and the author's of six to nine, show that regeneration of heads with more than five segments is not exceptionally rare. Smaller numbers in previous work may have been due to less favorable conditions.¹

The maximum number of segments now recorded for head regenerates in the family Lumbricidae is nine. With the exception of one pair of seminal vesicles and of spermathecae, both of which develop in connection with septum 9/10, reproductive organs in the Lumbricidae are in the region from x posteriorly. All of the evidence available still indicates that Lumbricids regenerate anteriorly only a pregonadal portion of the body. Regeneration, after amputation of the gonadal region, accordingly, is not sufficiently "complete" to enable an individual to reproduce. Although this has often been thought to be characteristic of earthworms generally, at present it appears to be applicable only to the Lumbricidae. In those representatives of other families that have been studied, regeneration of the gonadal region not only is possible but even usual (see Janda, 1926, for the Glossoscolecid *Criodrilus lacuum*, and Gates, 1941, for the Megascolecid *Perionyx excavatus*). The pattern of regenerative capacity, even with regard to this one matter, accordingly, is not uniform throughout the earthworms.

Such data as are now available with regard to segment number, and in particular "7 or 8" segments in a head regenerate at EL 30/31 (Table I), do not appear to support current ideas as to decline in number of head segments regenerated as level of amputation recedes posteriorly (Hymen, 1940, p. 519) and gradient of head regeneration (Liebmann, 1943, p. 601, Fig. 12).

New data given above as to the morphological nature of anterior regenerates agree with some hitherto overlooked in showing a region of definite bipotential regenerative capacity. On amputation within that region a worm may regenerate either a head or a tail. The individual variation in response to the same stimulus suggests a possibility of experimental modification of the nature of regenerates.

The region of bipotential capacity, according to the author's results, is small and bounded by 20/21 and 23/24. Previous work indicates the possibility of considerable extension of those boundaries. Involved in estimation of the posterior limit of cephalic regeneration are two regenerates at EL 30/31 and EL 50/51 (Morgan, 1899 and 1901). Both, it is important to note, were obtained after rediscovery of heteromorphosis. The cephalic nature of the first was proved from sections. The second, having five metamERICALLY normal segments, presumably was large enough to be easily and correctly identified. Level of the first amputation could have been from 31/32 to 38/39 (see note d, Table I), but was probably in re-

¹ Carpenter now reports obtaining in one series, seven six-segment, two seven-segment, and one eight-segment head regenerates at 10/11.

gion of 32/33–35/36. The other amputation, estimated to be at the middle of the body, could have been from 41/42–64/65 (see note d, Table I), but with probability of location at or even in front of 41/42. A level about midway between 30/31 and 40/41, i.e., 35/36, appears at present to be as good an estimate as is possible in the circumstances.

The anterior boundary for heteromorphic tails is extended to 18/19 by Dimon's results (Table I). However, some of her "uncertain" regenerates at 17/18–15/16 presumably had, in absence of all reference to monstrosity, similarities to caudal regenerates. Morgan also had a regenerate at 15/16 thought to be "possibly a new tail" (1902, p. 579). All this seems to warrant placing the anterior boundary provisionally at 15/16. It is also noteworthy that at several still more anterior levels, to 10/11, some regenerates were "imperfect" or "very imperfect," characterizations apparently applied also to regenerates later found to be caudal.

Gradient of segment number in heteromorphic tail regeneration appears, from the author's data, to be of an inverted V-shape rather than the even slope apparently anticipated by Morgan (1902, p. 577) from results obtained on small fragments.

Failure, in the author's experiments, of normal worms to regenerate at levels behind 54/55 was unexpected in view of the results obtained from substrates as small as L14S in *A. terrestris* (Korschelt, 1898, p. 80). Regeneration by tail regenerates from levels behind 54/55 (E49), and behind that level by substrates conditioned by a previous regeneration (E58), suggests a possibility that failures on normal specimens were due to unfavorable conditions.

Regeneration of heteromorphic tails anterior to 20/21 and of heads behind that level, and more important, of both heads and tails from the same levels, does not appear to be in accordance with Liebmann's hypothesis (1943) that specifically polarized, eleocytic aggregates in the coelomic cavities, a head aggregate in v-xx and tail aggregates behind 20/21, determine the nature of the regenerate.

SUMMARY

Posterior substrates of *E. foetida*, cut exactly at intersegmental furrows, regenerated homomorphic heads at levels 1/2–23/24, with equimery at 1/2–8/9 and hypermery (+ 1) at 4/5 and 8/9. Heteromorphic tails regenerated at 20/21 and from 23/24 to 54/55. Behind 54/55, regeneration of heteromorphic tails was obtained only from tail regenerates and substrates conditioned by a previous regeneration. Gradient of segment number in heteromorphic tails appears to be of an inverted V-shape.

Starvation for 70 + days did not inhibit regeneration at 5/6 and 6/7 but all regenerates were hypomeric.

Hypermery and hypomery provide an adequate explanation of the origin of symmetrical homoeosis.

Regenerative capacity in a region from 20/21 to 23/24 is characterized as bipotential since an anterior regenerate, in that region, may be a homomorphic head or a heteromorphic tail.

Review of previous work on *E. foetida* provides indications that the region of bipotential regenerative capacity is even more extensive, with anterior limit of heteromorphosis possibly at or even in front of 15/16 and posterior limit of homomorphosis in region of 35/36.

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FREE-ENERGY RELATIONS AND CONTRACTION OF ACTOMYOSIN¹

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There are two approaches to muscle. One is that of the physiologist, who studies function hoping to understand the nature and reactions of minute structural elements. The other is that of the biochemist, who studies minute structural elements hoping to understand function. The physiologist carefully preserves structure and subtle qualities; the biochemist wilfully destroys them. This destruction may go as far as the dissolution of the system into single molecules. This approach was that of the author's laboratory, which has shown that the contractile matter of muscle is built of two proteins, actin (F. B. Straub 1942, 1943) and myosin.

Destruction need not necessarily go that far. It may be limited to partial dissolution, which leaves the contractile matter and its geometry untouched, or it may simply consist in the disturbance of certain equilibria by thermal or chemical means. In these cases, it is still convenient to call the system "muscle," but it should be clearly understood that by using this word no attempt is made to confuse such a partial system with the whole living and intact machinery.

In themselves, neither myosin nor actin is contractile. If brought together in a suitable ionic milieu they unite to a complex: "actomyosin." According to the concentration and the nature of ions present, the actomyosin may be charged by the ATP and dissociate reversibly into its two components, or else it may be discharged and dehydrated excessively. If this reaction takes place in a heterogeneous suspension, the actomyosin is precipitated. Owing to its violence, this precipitation was termed "superprecipitation" to distinguish it from the weaker dehydration and precipitation induced by salts alone in absence of ATP. If this reaction takes place in an actomyosin gel, it will lead to excessive shrinking, syneresis. If the elongated actomyosin particles are oriented, the shrinking will be anisodiametric and the gel shrinks in the direction of the axis of the particles and expands at right angles to this direction. Actomyosin threads or muscle fibres, under these conditions, may become shorter and wider without changing their volume. If the reaction takes place in the muscle fibre where the elongated actomyosin filaments form a continuous system, the shortening will be able to do work by lifting weights, or develop tension under isometric conditions, and is usually called "contraction."

The study of these phenomena suggested (see Szent-Györgyi, 1947) that the

¹ This research has been sponsored by a grant from the American Heart Association.

contractile matter is built of functional units, "autones," and that contraction is an "all-or-none equilibrium reaction" of these autones, dependent on temperature.² Contraction, *i.e.* the dimensional change, in all probability, is secondary to another change in which charges are neutralized. The size of "autones" is independent of the colloidal particle size ($1-1.5 \times 10^6$ g) into which myosin breaks up on extraction, and can be expected to be much smaller than this latter. Supposing that the actual shortening is proportional to the number of reacting units, the relative number of charged and discharged units (that is the equilibrium-constant K) was calculated from the macroscopic length of the system.

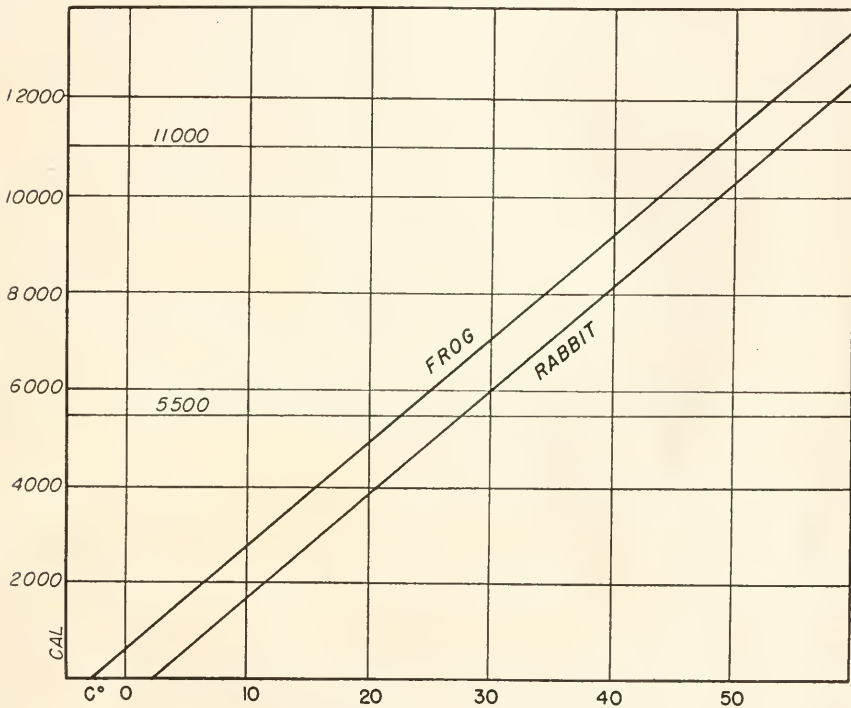


FIGURE 1. The ΔF curve of the contraction of extracted frog and rabbit muscle and of frog and rabbit actomyosin threads. Ordinate: ΔF in calories. Abscissa: temperature in centigrades (quoted from L. Varga).

From the temperature-dependence of K , the ΔF , *i.e.* the difference of the free-energy content of contracted and relaxed autones, was calculated. The final results of these calculations were summed up by L. Varga (1946) in a curve reproduced in Figure 1. The curve shows that the free energy of the system drops in contraction and that the extent of this drop depends on temperature. In the rabbit it reaches 11,000 calories at 53° C., is 7–8000 calories at 37°, and is 0 at about 0° C.

² Buchtal and Knappeis pointed out in 1943 that certain mechanical features of muscular contraction are in accordance with the assumption that the fibre is built of smaller units contracting in an "all-or-none" fashion.

In the frog the same values were reached at 5° C. lower. The curve shows that the relaxed state is the high-energy metastable state, the contracted state the low-energy stable state, contraction being a spontaneous process.

In the first part of this paper material and methods will be discussed. In the second part, the theory will be tested along different lines, and in the third part, the observations will be extended.

PART I: MATERIAL AND METHODS

Muscle is a very heterogeneous tissue. Not only are there different kinds of muscle (smooth, heart and cross-striated muscle), but there are considerable differences between the different muscles of the same sort within the same animal.

There is considerable difference in geometry between the various body muscles. In one muscle the fibres are parallel, while in others they follow a more complicated course, making evaluation of energy relations difficult.

There is considerable difference, also, in the composition of various muscles. The contractile matter, actomyosin, is in its relaxed condition a soft gel which could easily be damaged by mechanical injury were it not protected by connective material, fasciae, collagen fibres and a sarcolem. Muscles lying closer to the surface will need more protection, and in these we will find strongly developed connective material and sarcolem.³ An almost ideal material for the study of the contractile matter is the musculus psoas of the rabbit. This muscle lies sheltered in the body cavity, protected on one side by the vertebral column, and by the viscera of the belly on the other. Consequently, it contains very little connective tissue, and the sarcolem is poorly developed, which makes the elastic properties of the contractile matter come to the fore. It is built of very long, parallel fibres, stretching from one end of the muscle to the other—fibres which, owing to the poverty of connective material, can easily be separated. It is easy to secure from a medium-sized rabbit very thin fibre bundles 8–10 cm. long which, if necessary, can be decomposed into single fibres of this length. Though occasionally frog sartorius and rabbit m. gracilis were also used, the major part of the experiments reported here were performed on the psoas.

According to the theory outlined, contraction is a spontaneous process going hand-in-hand with a drop of free energy. Thus, contraction should occur spontaneously wherever the ATP-actomyosin system is present in a suitable ionic milieu, and the system should persist in the low-energy stable contracted state. This is actually what happens any time we add ATP to an actomyosin gel or to muscle extracted with water. In the intact resting muscle, however, we find ATP in an active form, linked to actomyosin (see below), but still the system does not contract—contraction being inhibited by some unknown mechanism. If we want the muscle to go over into the contracted state, we have to abolish this inhibition. In the intact muscle this can be achieved by an electric shock or a "wave of excitation." These actions are fleeting and depend on subtle qualities of muscle, on "excitability," which makes them unfit for our present purpose. In order to study equilibria of energy relations, the inhibition had to be removed permanently and uniformly

³ Ramsey and Street (1940), working with single fibres of the musculus semimembranosus of the frog, found the elastic properties of the contractile matter in resting muscle entirely covered up by the elastic properties of the sarcolem.

throughout the whole mass of the muscle, and the whole contractile matter made to go over into and remain in the contracted state. Poisons like caffeine, quinine, monojodo-acetic acid or chloroform, known to produce contracture, were found unsatisfactory because the tensions developed are very small, showing that only a small fraction of the contractile substance is at any time in the contracted state.

A satisfactory method of abolishing inhibitions is freezing with subsequent thawing, which method also has the advantage that the muscle can be kept in the frozen state, packed in dry ice, for days with undiminished contractility.

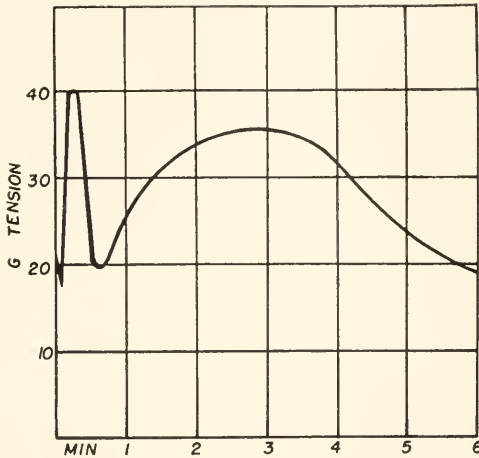


FIGURE 2. Isometric contraction of the frozen sartorius of the frog on thawing. The frozen muscle was immersed into Ringer of 20° C. at 0 min.

The experimental procedure was the following: The rabbit was killed by decapitation, quickly skinned, eviscerated, and the front and the sidewalls of the belly cut off. This exposed the psoas which was liberated from its surroundings. The muscle was decomposed into smaller bundles by punching it through with a small forceps with closed tips and moving the forceps up and down while the index finger of the other hand kept the muscle somewhat lifted. If necessary, ligatures were put on the two ends of the muscle strip. Owing to the poverty of connective tissue, the muscle is rather soft and is easily cut through by ligatures. For this reason, relatively soft and thick threads were applied in dry condition (pearl cotton No. 5).

If frozen strips were desired, fibre bundles of 2–3 mm. diameter were secured, placed on a celluloid ruler (to which the muscle does not stick), stretched to their rest length, the ligature being fixed by artery clamps. Then the strips were covered with freshly powdered dry ice. The strips used were mostly of the thickness of an average frog sartorius, weighing about 40 mg. per cm. Since at higher temperatures the muscle, after freezing, is rapidly damaged, it is important that it should reach temperature equilibrium quickly. So if experiments had to be performed above 30° C., even thinner strips were used weighing 25 mg. per cm.

On thawing, the frozen muscle, if containing the physiological amounts of ATP, contracts rapidly and develops maximal tension.

There are two phenomena which tend to disturb measurements. If the frozen muscle is suddenly placed into Ringer of room temperature, one side may thaw faster than the other and contract suddenly, which causes strong bending which damages the fibres. For this reason, the muscle was allowed to thaw first in 0° Ringer before being transferred into a warmer Ringer of more than 15° C. The first movement of the lever indicates complete thawing which may take place in ten to sixty seconds.

The contraction, elicited by the freezing and subsequent thawing, is developed relatively slowly while the sudden change in temperature may act, in itself, as an impulse and elicit a fast contraction. In this way, in frog muscle, a double peak is obtained, the second of which is mostly lower than the first (Fig. 2). In rabbit psoas this double contraction is less pronounced but still present. Below 10° C. "excitability" is low and the two waves fuse. At higher temperatures they can be made to fuse by making the temperature change less sudden by allowing the muscle to thaw in Ringer of 0° C. before applying the higher temperature. Tension given to the muscle also promotes fusion of the two peaks. The same is favored, also, by a thinner diameter. Correct values of maximal work or tension can be obtained only if the two contractions fuse into one.

In order to show whether an observed effect was actually due to an interaction of ATP and actomyosin, the latter had to be prepared free of ATP. The effects observed on addition of ATP could then safely be ascribed to an interaction of the protein and the nucleotide.⁴

Thus muscle fibres had to be prepared, free of ATP, and made permeable to this substance, without destroying the actomyosin structure. In earlier work this was done by extracting the muscle with water. In pure water, however, even at low temperature, muscle fibres preserve their full contractility only for a short time.

Satisfactory results were obtained by employing a 50 per cent solution of glycerol. The fibre bundles, once extracted, can be preserved for weeks in this solvent at -20° C. with undiminished contractility. The psoas was decomposed *in situ* into fibre bundles of about one millimeter in diameter. A thin stick was laid alongside, and four or five such bundles were tied to it at both ends and cut out. In this way straight fibre bundles of rest length and attached to the stick were secured. If bundles of equilibrium length were desired, only one end of the bundle was fixed to the stick and the other end cut, whereupon the muscle contracted to its equilibrium length. Then the other end of the bundle was fixed to the stick. In order to measure the difference between rest length and equilibrium length, a ligature was put on the free end of the bundle before cutting it, and the distance between the two ligatures was measured before and after cutting.

The bundle, tied to the stick, was placed into 50 per cent glycerol of 0° for twenty-four hours. Then the two ends of the muscle with the ligatures were cut off, whereby the muscle, detached from the stick, fell into the single bundles. The muscle was left in this condition for another day at 0° in 50 per cent glycerol and then transferred in this solvent into the deep freezer kept at -20° C.

⁴ Threads prepared from actomyosin are unfit as material for any experiment in which tensile strength is involved, since on extraction the continuous actomyosin filaments present in muscle are broken up, and actomyosin threads contain only their fragments. As will be shown later, one of the actions of ATP is to enable the actomyosin particles to slip alongside one another. Therefore, if an actomyosin thread is loaded or subjected to tension, and ATP is added, the actomyosin particles will contract, as they do in muscle, but they will also slip, and in spite of the contraction (observable in unloaded threads), the system will lengthen. This lengthening has led Buchtal, Deutsch, Knappeis and Petersen (1947) as well as Astbury, Perry and Reed (1948) to the erroneous conclusion that phenomena in actomyosin threads are fundamentally different from those in muscle.

The muscle in 50 per cent glycerol is too stiff to be decomposed into smaller bundles without straining, which causes the bundles to curl up. In water the muscle is too soft. For this reason, before the experiment, the bundles were transferred from 50 per cent glycerol to 20 per cent glycerol for an hour or so and decomposed here to the desired diameter, mostly into strips of 0.2 — 0.5 mm. diameter.

The psoas is built of smaller fascicles, and it is well to follow the outlines of these preformed bundles in decomposing the muscle. The dissecting was done by means of a pair of fine tweezers, used by watchmakers. The ends of the bundles are caught with these tweezers. By pulling them apart, the bundles can readily be separated. Before being subjected to experiment, the fibre bundles were examined under the microscope for continuity.

The experiments were performed in Ringer containing 0.001 M $MgCl_2$. In all experiments glass-distilled water was used because of the deleterious action of copper usually present in common distilled water.

Glycerol-treated fibre bundles of rest length and of 0.1 — 0.2 mm. diameter, if placed into a 0.25 per cent ATP solution, contract rapidly. Diffusion being the limiting factor, the rate of contraction depends also on the diameter. Unloaded fibres contract at room temperature to one-fourth or one-fifth of their rest length. If connected to the isometric lever, on addition of ATP they develop tension comparable in intensity to that developed by intact muscle on maximal excitation. If loaded they will also lift weights isotonicly, similarly to intact muscle fibre bundles of similar dimensions.

This contraction of glycerol-treated muscle fibres under influence of ATP is one of the most striking biological phenomena and is very suitable for classroom experiments. Instead of ATP a freshly prepared boiled muscle juice may be used, or an ATP solution may be used prepared by elution of dried, alcohol-precipitated muscle. A smallish rabbit will provide material for a big class. Most of the experiments reported here were done by simple means and are suited for classroom experiments. Some of them have been repeated by the physiology class at the Marine Biological Laboratory at Woods Hole.

In several experiments the maximal total amount of work had to be measured. Theoretically, this can be done in the following way: The muscle is connected to the isometric lever, made to contract, and the tension is measured. Then the muscle is allowed to shorten slightly and made to contract, and the tension measured, etc., till the muscle has contracted maximally and develops no more tension. If the length is plotted against tension, the area between length and tension represents the total amount of work. Such an experiment was performed on the frozen sartorius after thawing. Its result is schematically reproduced in Figure 3, where the hatched zone is the total amount of work. This experiment is a rather difficult one and can be performed only with limited material and under specific conditions. In the rabbit psoas, contractility is lost after thawing even at 0° C. in fifteen minutes, which makes the experiment impossible. In the frog the experiment can be done at low temperatures only, contractility being lost rapidly at higher temperatures.

A simpler method had to be found which could be applied in any material in a wider range of temperatures. Two such methods are suggested by Figure 3. In this figure the total amount of work is equal to the area BDF, which is one-half of the area BDFH, and the double of the area CDEG. Accordingly, we could measure the total amount of work in two different ways: (1) by measuring the maximum tension developed (DF) and multiplying it by the amount of maximum shortening

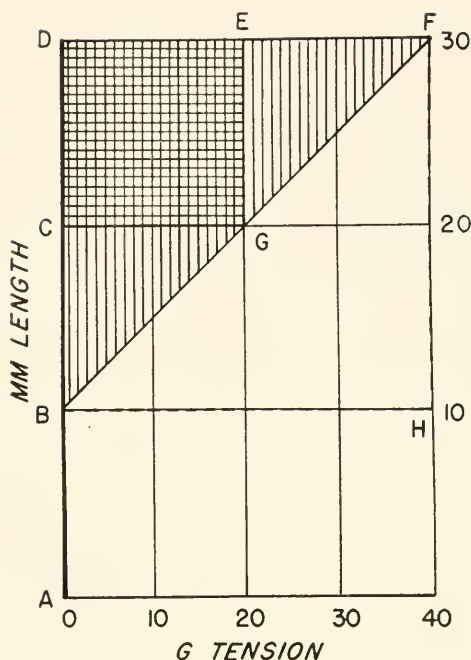


FIGURE 3. Schematic representation of muscular contraction. (Sartorius of the frog, Temp. 2.5° C.) AD = initial rest-length, DF = maximum tension, AB = length of maximally contracted muscle, unloaded. Area BDF = total amount of work. BF = length-tension diagram of the excited muscle.

of the unloaded muscle (BD), then dividing the result by two. Psoas strips, after freezing and thawing, contract at room temperature by two-thirds of their rest length.

The formula will thus be $\frac{2/3 \text{ lt}}{2} = 1/3 \text{ lt}$ (1 = rest length, t = maximum tension).

A. V. Hill (1913) developed a similar formula which in his case (frog muscle excited electrically at 0°) was $1/6 \text{ lt}$. (2) Load the muscle with the weight corresponding to one-half of the maximum tension (DE), measure the distance by which the weight is lifted (EG), and take the double of the product of these two magnitudes (the area CDEG). The product will be the biggest if the weight is just one-half of the maximum tension, but a small deviation from this value will not cause a considerable error making the area bigger in one dimension and smaller in the other.

The first will be called the "isometric," the second, the "isotonic" method. Both methods may be criticized as to their exactness. The object of the present research is not to obtain exact numeric values, but to obtain information about the basic truth of the theory outlined.

PART II

Observation on heat contracture

According to Figure 1, the ΔF , i.e., the free energy spent by the single units, rises with increasing temperature. The free energy of the phosphate bond in ATP

is 11,000 calories (Meyerhof, 1944) and according to the theory discussed, this energy is needed for relaxation. According to the curve in Figure 1, the expenditure of energy in contraction reaches 11,000 calories at 47° in the frog and at 53° in the rabbit, and exceeds 11,000 calories above these temperatures. If the theory is correct, therefore, the muscle should be unable to relax at these temperatures and should persist in the fully contracted state.

In the frog, experiments were performed in the following way: the sartorius of *Rana pipiens* was provided with a ligature at both ends, was excised and loaded in one series with 2 g. (Fig. 4, circles) and in another series with 20 g. (triangles). The length between ligatures was measured and the muscle dipped into Ringer solution of varying temperature. Above 40° a rapid contraction ensued which was measured at the end of the second minute. The contraction reached its maximum at 47° C. The muscle remained in this maximally contracted state. The gradual lengthening at higher temperatures is due to the denaturation which takes place above 47° C. rather rapidly.

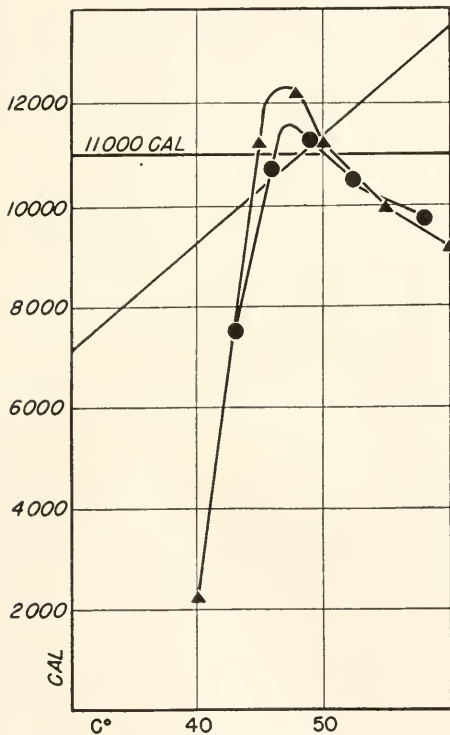


FIGURE 4.

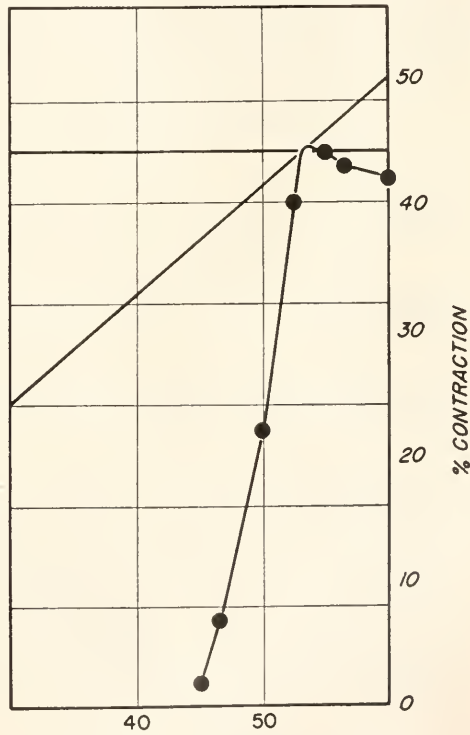


FIGURE 5.

FIGURE 4. Heat contracture in the frog sartorius. The coordinate net corresponds to the right-hand side of Figure 1. The sloping straight line is the ΔF curve, and corresponds to the left-hand side ordinate of Figure 4. The points mark per cent of shortening and relate to the right-hand side ordinate of Figure 4. Abscissa: temperature in centigrades.

FIGURE 5. Same as Figure 2. Strips of the musculus gracilis of the rabbit.

In the rabbit, experiments were performed in a similar way with the smaller weight (Fig. 5). Experiments were performed with strips of the musculus gracilis similar in dimensions to the frog sartorius. These strips were cut parallel to the fibres. The experiment was performed soon after the animal's death. The results were similar to those obtained in the frog. In both cases the muscle went into permanent maximal contraction at the temperature where the ΔF curve cuts the 11,000 calorie level, as demanded by the theory.

The fact that maximum and permanent contracture was reached only where the expenditure of F was 11,000 shows that the transference of energy from ATP to the contractile system goes without considerable loss.

This heat contracture must not be confused with the shortening of muscle due to heat denaturation. If a muscle is immersed into Ringer of 70° C. an extensive shortening is produced which is not due to the mobilization of the normal mechanism of contraction, but to denaturation. The difference between the two processes can easily be demonstrated. If the muscle is stored a few hours after death at room temperature or overnight in the ice box, the ATP disappears. No rapid contraction will be obtained at 53° in this muscle, but shortening will still be obtained at higher temperatures at which rapid denaturation is produced. This denaturation manifests itself, also, by a turbid appearance. The basic difference between the contraction obtained in the presence of ATP at 53° in the rabbit or 47° in the frog, due to the mobilization of the normal mechanism of contraction, and the shortening produced at higher temperatures and due to denaturation, can be demonstrated, also, by connecting the muscle to

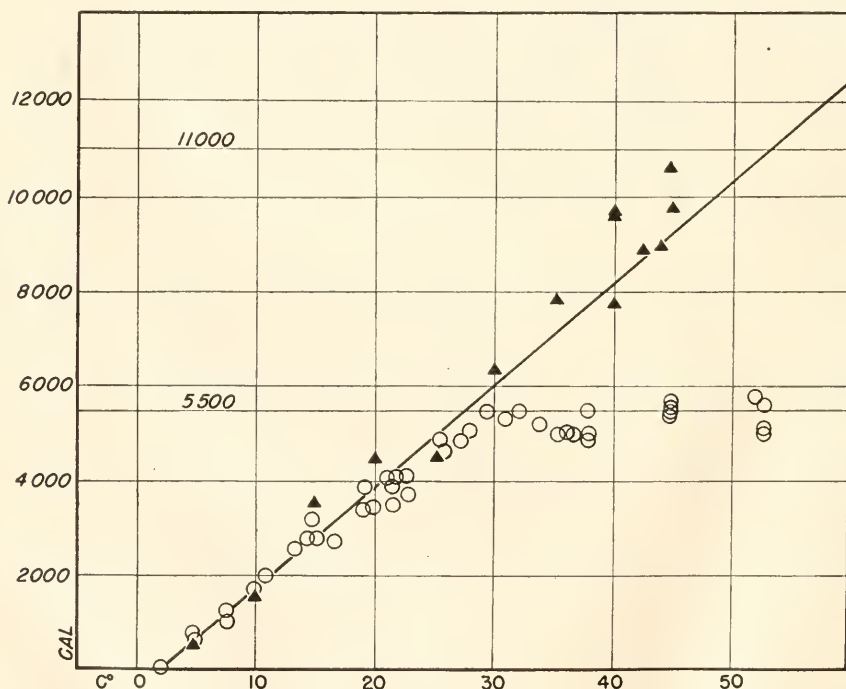


FIGURE 6. The work performed by strips of rabbit psoas, after freezing and thawing, at varied temperature, calculated for 35,000 gm. myosin present. The coordinates are identical with those of Figure 1. The sloping straight line is the theoretical ΔF curve. Triangles: isotonic measurement. Circles: isometric measurements.

the isometric lever. While the theoretical maximal tension is produced in the first case, scarcely any tension is developed in the latter. The gradual lengthening of the loaded muscle above 53 resp. 47° C. is evidently due to the denaturation. This denaturation in the rabbit sets in very rapidly above 53°.

Total work in the psoas

Free energy being, by definition, that amount of energy which can do work, the most direct way of testing our ΔF curve is the measurement of the total work at different temperatures.

The amount of work was measured by both the isotonic and the isometric method and the results are reproduced in Figure 6. The psoas strips were loaded or connected to the isometric lever in the frozen condition, a moderate tension being given to the lever. The work done was expressed in calories and calculated for 35,000 g. of myosin. In these calculations the average myosin content of muscle (8 per cent) was taken into account, though it is probable that owing to poverty in connective matter, the psoas contains somewhat more myosin. In order to find out the quantity of myosin present, the muscle was weighed immediately after the measurement was finished, its ends with the ligatures having been cut off.⁵

The results of the isotonic experiments are marked in the figure with triangles. No measurements could be taken above 45° C. owing to the great sensitivity of the muscle to high temperatures after freezing and thawing. As will be seen, the agreement with the ΔF curve is satisfactory and pleads for the basic truth of the theory.

The values obtained in the isometric measurement are marked with circles. As the figure shows, at lower temperatures the agreement of the experimental values with the ΔF curve is satisfactory. This is true, however, only up to the 5500 calorie level, at which it shows a break to become parallel to the abscissa. This means that the maximum of tension is reached at 28° C. and increases no more if the temperature is raised.

This inability of the muscle to produce the expected tension at these higher temperatures is one instance of the so-called Fenn effect, named after its discoverer who found (1923) that the work done by the muscle depended also on the sort of job the muscle had to do.

The isometric method of calculating total work is based on the assumption that the length-tension diagram is a straight line. It is evident that in this region, where the isometric and isotonic curves differ, the l-t diagram cannot be straight and the method cannot be used. If the results calculated by this method are reproduced, this is because they nevertheless show the fact that on raising the temperature the tension developed remains constant.

The actual l-t diagram of the psoas can be found in this region by loading the frozen muscle strips with different weights, bringing them to the temperature in question, and measuring the maximum distance to which the weights are lifted. The results of such an experiment, calculated for the same weight of muscle, are reproduced in Figure 7.

The ΔF curve of frog muscle

Varga's ΔF curve admits but a very small expenditure of energy for the frog muscle at 0° C. (600 cal. of the 35,000 gm. unit), which means that at this tem-

⁵ After freezing and thawing, contracted muscle rapidly loses weight by pressing out water. This loss may exceed 30 per cent, and is in agreement with the assumption that contraction is connected with loss of charge and hydration.

perature this muscle should be capable only of very feeble motion. It means, also, that the efficiency of the muscle would be exceedingly low because every autone would have to split one high-energy phosphate and pay 11,000 calories for the 600 calories spent.

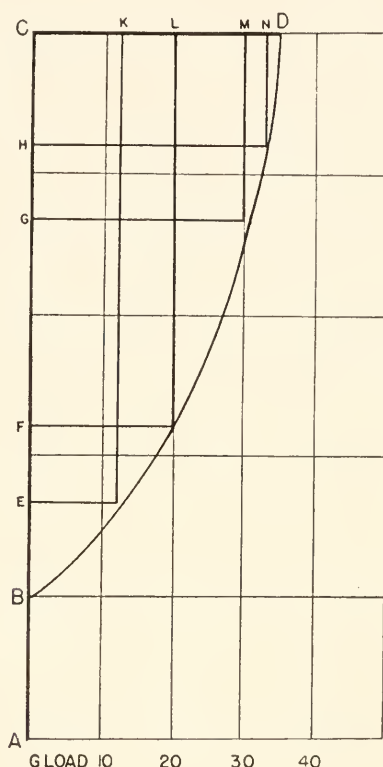


FIGURE 7. Length-tension diagram of strips of the psoas excited by freezing and subsequent thawing at 40° C. AC = length of the muscle; CH, CG, CF and CE are shortening with the weight CN, CM, CL and CK. The curved line connecting the corners of the squares (representing work done) is the l-t diagram.

As pointed out by A. V. Hill in the discussion following the author's lecture at the International Physiological Congress at Oxford (1947), frog muscle at 0° C. is capable of rather strong motion if excited strongly by direct stimulation. Hill had shown previously (1913) that not only is the tension developed by frog muscle at 0° C. rather high, but also the efficiency, which reaches 40 per cent (1939). The author was able to convince himself of the correctness of A. V. Hill's statements. Results of a few experiments on this line are reproduced in Figure 8. They show that the muscle actually spends much greater amounts of energy than allowed by the ΔF curve. The average expenditure around 0° was found to be 4500 calories, which corresponds to a 40 per cent efficiency if the 4500 calories are paid for by

the 11,000.⁶ This agreement with Hill's results shows that freezing and thawing yields results similar to electric excitation.

The reason for the discrepancy between Hill's and Varga's results obviously was to be sought in the different nature of the material. Hill worked with intact muscle, Varga with muscle extracted with distilled water and frozen, or with actomyosin threads. It is easy to believe that in the animal the behavior of the actomyosin system is adapted to life at low temperature by some sort of regulation no longer present in extracted muscle or actomyosin.⁷ This assumption would become acceptable if it could be shown that the ΔF curve of the whole muscle can be transformed into the type of Varga's curve by substances which are known to abolish physiological regulations.

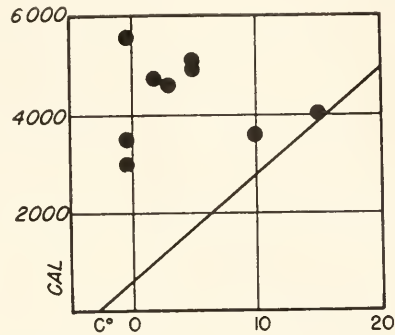


FIGURE 8. Work performed by the frozen sartorius after thawing, calculated for 35,000 gm. of myosin present. Coordinates correspond to lower left corner of Figure 1. Sloping straight line: ΔF curve.

Narcotics, at high concentration, inactivate many physiological mechanisms. Most of them also damage the contractile matter. Chlorated paraffins, however, like ethyl-chloride or chloroform, have no harmful action on actomyosin.

The sartorii of the frog (*Rana pipiens*) were exposed, and provided at their ends with ligatures; the distance between ligatures was noted. The muscle was excised, fixed at its original length and placed for five minutes in Ringer of 0° C., saturated with chloroform. Then the muscle was covered with freshly powdered dry ice and frozen. Its working capacity was measured in the isotonic and isometric experiment at varied temperatures. Also, the Ringer in which the muscle was made to thaw and contract was saturated with chloroform. If the work was measured above 15° C., the muscle was connected to the lever or the weight and allowed to thaw first in Ringer of 0° C., saturated with chloroform, and then transferred to the Ringer of higher temperature.

⁶ When the work done by the right and left psoas was compared at slightly varied low temperatures (e.g. 0° and 3° C.), the muscle was found to have a greater ΔF at higher temperature; the slope of the resulting ΔF curve cut the abscissa at about -30° C., and the 11,000 cal. level slightly under 47°. This suggests that the ΔF curve of intact frog muscle is similar to the curve of actomyosin, but has a different slope.

⁷ The experiments were performed between November and January, and thus winter frogs were used.

The work done, and thus the free energy spent, was calculated for 35,000 gm. of myosin. The results are reproduced in Figure 9. The isotonic measurements could not be extended further to the right, above 40° C., the frozen muscle being damaged rather readily by higher temperatures. The action of the chloroform is reversible, and if the chloroform is washed out the work done at lower temperatures increases.

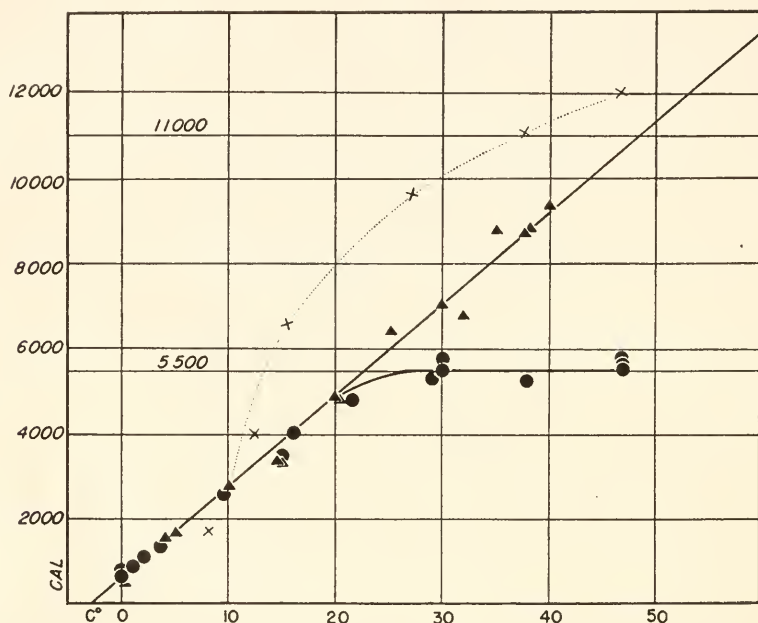


FIGURE 9. Work performed by the sartorius of *Rana pipiens* under influence of chloroform. The curve is analogous to Figure 5. Crosses: isotonic experiment with weights applied prior to freezing.

As the figure shows, the results obtained are similar to those obtained in the rabbit; the curves have the same shape but lie 5° lower. Here, again, the isotonic curve becomes asymptotic on reaching the 5500 calorie level.

In a series of experiments the muscle was loaded prior to freezing with the weight it was to lift later. The results are marked in Figure 9 by crosses connected by a dotted line. No correction was made for the elastic tension, but this correction would be smaller than the actual deviation from ΔF curve, which suggests that the expenditure of energy depends also on the tension which has to be overcome. It was observed repeatedly that the sartorius developed higher tension after freezing and thawing if stretched previously for a short while.

To sum up the experience with frog muscle, we can state that the ΔF curves of whole frog muscle and extracted frog muscle are different, the latter being identical with the ΔF curve of actomyosin threads. By treatment with chloroform, the curve of the whole frog muscle can be transformed reversibly into a curve similar to that obtained by Varga in his extracted material. This brings out the point

that the ΔF curve of actomyosin can be greatly modified by accompanying substances, and opens the possibility of adapting the contractile material to different physiological functions. Actomyosin is not a sharply defined substance and is accompanied by other different substances, proteins and lipins which actually make part of the system, and it is not surprising to find that systems containing different substances may have different ΔF curves. Ionic equilibria, disturbed by extraction, may also contribute to shaping the ΔF curve.

In the glycerol-treated psoas, immersed in 0.2 per cent ATP dissolved in Ringer, the ΔF curve, obtained by the isometric method, cuts the abscissa if extrapolated at -10°C ., while if extrapolated towards high temperatures, it cuts the 11,000 calorie level somewhat above 50° and has thus a different slope than untreated muscle.

Thermodynamic reversibility

One of the most important implications of the ΔF curve (Fig. 1) is the thermodynamic reversibility of contraction, which means that the energy spent by the single units in contraction is a function of temperature on which it depends in a reversible way. It should thus be possible to increase or decrease the tension of the contracted muscle solely by variation of the temperature.

Owing to secondary complications, not every material is suitable for this demonstration. The rapid loss of ATP and contractility in the rabbit muscle, after freezing and thawing, rules out this material. In the intact frog muscle, as shown, the effect of temperature is compensated. We can expect to be able to demonstrate

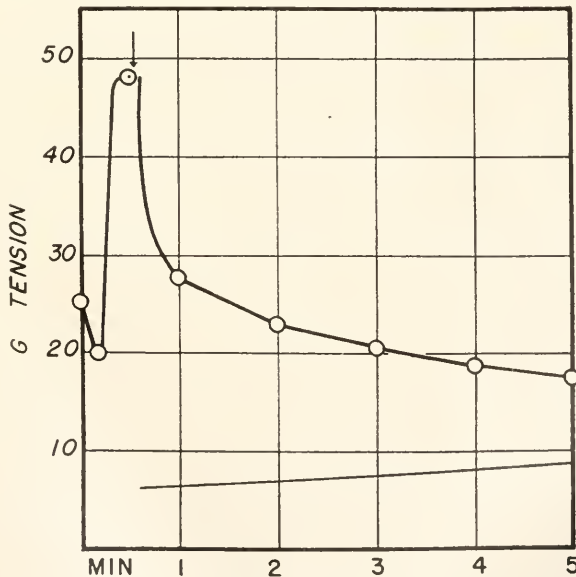


FIGURE 10. Tension developed by the frog muscle, treated with chloroform, at 13 and $0-1^{\circ}\text{C}$. At the arrow the warmer Ringer was substituted by the cold one. Sloping straight line: tension demanded by the ΔF curve.

thermodynamic reversibility in the frog muscle treated with chloroform, or in extracted strips of the psoas immersed in a solution of ATP.

Figure 10 illustrates a result obtained with frog muscle. The sartorius was treated with chloroform and frozen, connected to the isometric lever and dipped into chloroform Ringer at 13° C. After a short negative phase, usually seen in such conditions, the muscle rapidly contracted developing 48 gm. of tension. As the maximum was reached (26 sec.) the Ringer was exchanged for another chloroform Ringer of 0° C. The muscle suddenly relaxed. During the experiment the temperature of the Ringer rose 1° C. The sloping line in Figure 10 shows the theoretical tension of the muscle demanded by the ΔF curve. As can be seen, the tension of the muscle asymptotically approaches this line. The control experiment done with the other sartorius of the same frog showed that if the temperature is kept constant at 13° C., the tension remains high after a slight initial depression and does not fall more than a few per cent in five minutes.

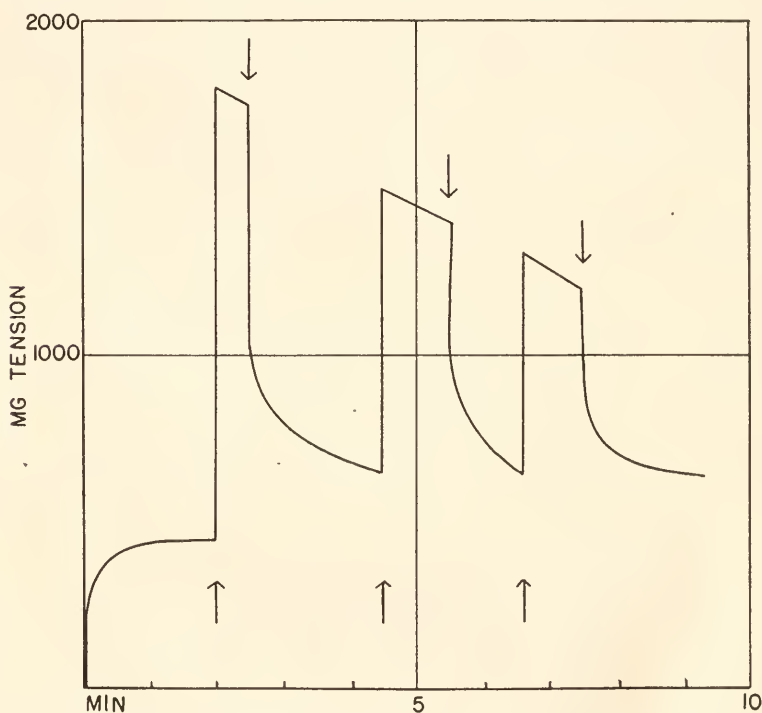


FIGURE 11. Tension developed by extracted psoas fibres in ATP at 25° and 1° C.

The following experiment, reproduced in Figure 11, illustrates thermodynamic reversibility in a fibre bundle of the psoas, treated with glycerol: The fibre bundle (42 mm. long and 0.5 mm. thick) was connected to the isometric lever, a tension of 200 mg. was given, and the muscle immersed in Ringer of 1° C. Then the Ringer was exchanged for another Ringer of the same temperature containing 0.2 per cent ATP. The tension rose to 450 mg. At the arrow pointing upwards, the

fluid was exchanged for an identical solution of 25° C. The tension was noted at once and found to be 1800 mg. A reading was taken every thirty seconds and the warm ATP Ringer was exchanged for the cold one (arrow pointing downwards). A reading was taken at once and subsequently every thirty seconds. At the arrow pointing upwards, again, the warmer Ringer was introduced, etc. As can be seen in the figure, the change in tension is immediate and reversible. As the experiment went on, the muscle gradually lost contractility.

The measurement of the diameter has no pretense of accuracy. If the tension is calculated from the final measurement at 25° for one cm.², a tension of 2½ kg. is obtained, which shows that the tension developed by a glycerol-extracted muscle under the influence of ATP is of the same dimension as the tension developed by an intact muscle under the influence of maximal stimulation.

PART III

Elasticity of the resting psoas

The fresh, resting psoas shows a moderately high elasticity, as demonstrated by the following experiment (Fig. 12): A strip of the psoas of the freshly killed rabbit was connected to the isometric lever, weight 56 mg., rest length 78 mm. (RL in fig.), equilibrium length (EL) immediately after excision, 62 mm. The muscle was slowly stretched, its length being increased by one millimeter in five seconds. After the rest length was reached, the muscle was released for a few seconds and then its equilibrium length measured. This was done by straightening the muscle out, measuring its length, and then applying a tension of 200 mg. and measuring the length again. The difference in length in both measurements was usually 2 mm. In the figure the average of these two measurements is given. After this measurement was completed, the muscle was stretched to the length from which it was released. This stretching was roughly twice as fast as the stretching before. Then the muscle was stretched further at the original lower rate and the procedure repeated after every 5 mm. of additional stretching till the muscle broke. The muscle was kept during the experiment in a wet chamber, immersed in a water-bath of 0° C. In the figure the gradual stretching is symbolized by the upper straight line which refers to the ordinate (mm). The corresponding equilibrium length is reproduced in the middle curve. The single points of this curve lie under the point of the upper curve from which the muscle was released. The lowest curve shows the tension developed on stretching and refers to the ordinate, the numbers of which mean gram-tension in this case. The lowest straight line simply shows a slope of 45° and means that if the curve of tension is parallel to this line, the muscle obeys Hooke's law.

The upper line shows that the muscle broke when extended to 173 per cent of its equilibrium length, and the middle curve shows that this extension was elastic in the whole range of measurements. The middle curve illustrates the well known fact of the poor reproducibility of the equilibrium length. The lowest curve shows that up to the rest length the contracted units can be stretched practically without resistance, but begin to develop resistance at this point. If the muscle were *in vivo* at its equilibrium length, it could develop no tension at the beginning of contraction; if it were tensed any more, it would be spastic.

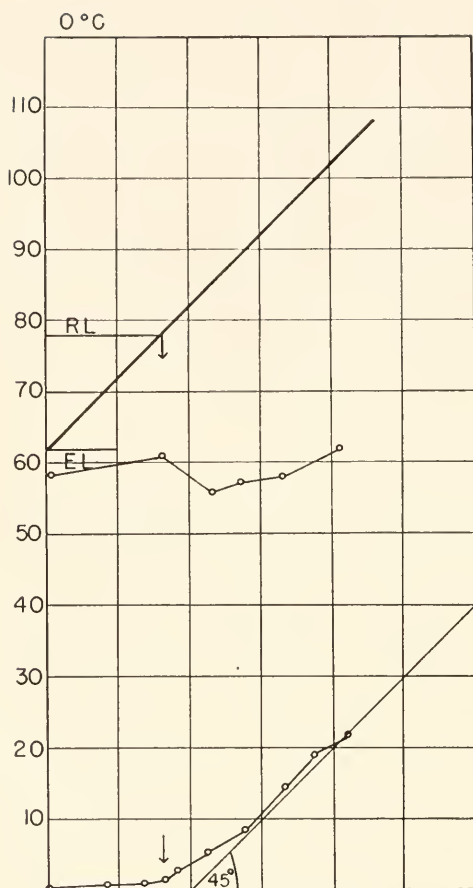


FIGURE 12. Length-tension relations of the psoas at 0°C . (see text).

In Figure 13 an identical experiment is reproduced, performed at 23°C ., weight of the muscle 130 mg.

In this experiment, the muscle broke when extension reached 190 per cent of the equilibrium length. As the middle curve shows, this extension is, at its higher degrees, not completely reversible, and the elastic part of the extension is but 163 per cent of the equilibrium length. The middle curve shows the rest length to be better reproducible at this temperature. The lowest curve again shows the contracted units to be extensible at the beginning with practically no resistance. The middle part of the curve obeys Hooke's law; the upper part shows excessive tension. The transition from the region of low tension into the Hooke region is rather sharp and corresponds to the rest length. If the relaxed units contract, the tension developed will be proportional to the contraction from the beginning which makes precise motion possible. On the other hand, having practically no tension, they will not impede the motion of their antagonist.

The muscle obeys Hooke's law up to one-half of the maximum of tension. At the point where it begins to develop excessive tension, stretching begins to be inelastic, causing slipping and permanent damage to the muscle.

Elasticity, ATP, and the slope of the ΔF curve

Freshly isolated strips of the psoas show high elasticity. After the death of the animal, its ATP gradually disappears in a few hours' time, as shown by M. Borbiero in a separate paper (pp. 162-7, this issue). Parallel to this disappearance of ATP, the elasticity of the muscle declines, and if the muscle is excised four hours after death, it will usually be found entirely inelastic. On stretching, the maximum tension is developed at once, and the muscle tears without considerably increasing its length.

The question arises whether the high elasticity of fresh muscle is actually due to the ATP present, and whether the disappearance of this elasticity can actually be attributed to the decomposition of this nucleotide. It can be shown that this is

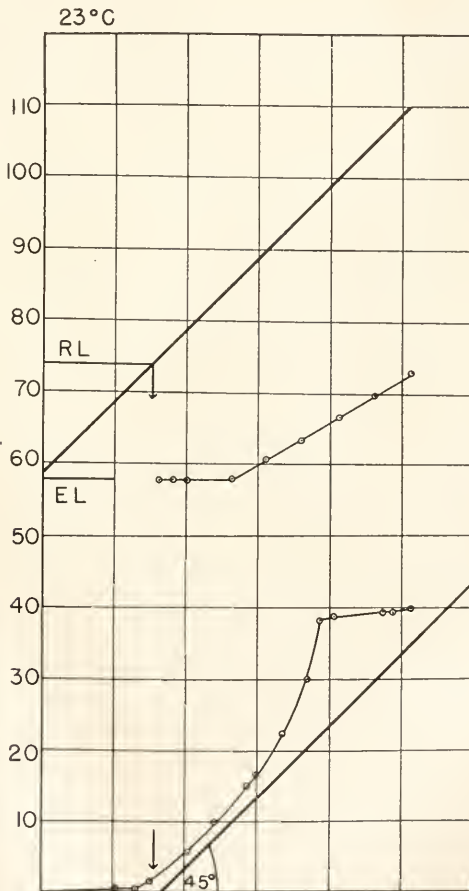


FIGURE 13. Same as Figure 12, at 23° C.

actually the case: if strips of the psoas are extracted at equilibrium length with 50 per cent glycerol, they are found to be entirely inelastic. At 0° , in Ringer, they cannot be stretched at all without breaking, and even at 13° C. extensibility does not exceed two per cent. If, however, 0.2 ATP is added to the Ringer, the muscle again becomes extensible. Using fibre bundles of 0.3–0.4 mm. diameter, the muscle could readily be stretched at 0.5 – 1° C., on an average to 145 per cent of its rest length.⁸

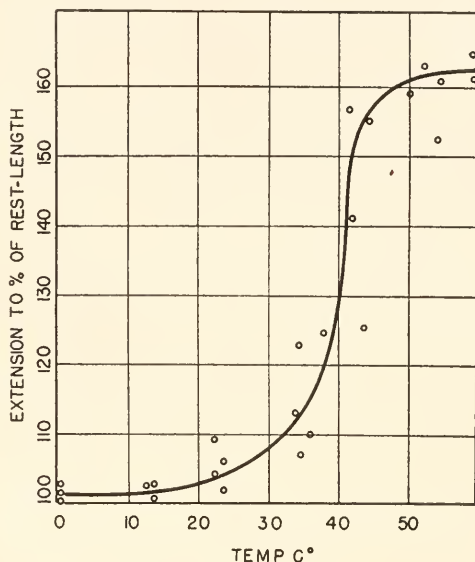


FIGURE 14. Extensibility of extracted psoas fibres at varied temperature. 100 per cent of rest length (abscissa) means that the fibres are not extensible.

The extensibility of the extracted muscle, in absence of ATP, is a function of temperature (Fig. 14). At 0° the muscle is practically not extensible; extensibility rises slowly with increasing temperature, rising rapidly at body temperature. The extensibility at higher temperatures, up to 53° , is not due to denaturation, as shown by the relatively big force needed for extension. Denaturation rapidly sets in at a somewhat higher temperature, 54 – 55° , where the muscle soon becomes plastic, offering practically no resistance to stretching.

This shows that, in absence of ATP, the actomyosin particles in muscle are surrounded by unbalanced forces which link neighboring particles together and make it impossible for them to move relative to one another, turning the system into a rigid, inelastic gel. They are counteracted by heat-agitation.

The extensibility of muscle at low temperatures in presence of ATP shows that the elasticity of muscle actually depends on its ATP, which must be present in the resting state linked to the actomyosin. The interdependence of ATP and elasticity also shows that in the psoas the elastic properties observed were predominantly

⁸ These results are in agreement with previous findings of Th. Erdős (1943) on the relation of ATP to rigor mortis.

those of the contractile matter, actomyosin, and were not due to the sarcolem or the connective tissue present, no specific reaction being known to take place between these latter and ATP.

It is evident that free energy is needed to abolish the cohesive forces linking the particles together, and if this is achieved by ATP, so it is also evident that part of the free energy of the ATP-actomyosin system will have to be spent in this reaction. The free energy spent in this reaction will manifest itself in the stability of the link between ATP and the actomyosin. The free energy needed to make actomyosin elastic will decrease with increasing temperature, actomyosin becoming more elastic by itself on elevation of the temperature. This expectation is in agreement with the results of Mommaerts (1941-1942) who found that at low temperatures, the dissociation constant of the actomyosin-ATP complex was exceedingly low. F. B. Straub (1941-42) found that the binding of pyrophosphate to actomyosin greatly depends on temperature, being strongest at the lowest temperature.

We can thus conclude that the free energy of the actomyosin-ATP system is spent in two successive steps. In the first step the ATP is linked to the myosin, cohesive forces are abolished, and a new system is formed in which particles are rendered capable of relative motion, contraction or extension. The free energy spent in this reaction, henceforth called "Reaction I," will decrease with increasing temperature.

The second step, "Reaction II," entails the dimensional change called "contraction." In the resting muscle, we find the actomyosin-ATP system in the state of Reaction I, but Reaction II is inhibited by some unknown mechanism. This inhibition is abolished by "excitation" which causes Reaction II to take place. In the glycerol or water-extracted muscle suspended in ATP this inhibitory mechanism is no longer present, and Reaction I is followed spontaneously by Reaction II.

It is evident that Reaction II can only spend the free energy unspent by Reaction I, which may be involved in the slope of the ΔF curve of contraction (Fig. 1).

Since actomyosin devoid of ATP-ase activity can still contract, as shown by Buchthal, Deutsch, Knappeis and Petersen (1947), we can conclude that no phosphate is liberated in Reaction I or II, and the whole loss of free energy of the system takes place without splitting of high-energy phosphate links.

The increase of extensibility of actomyosin under the influence of ATP was the first known specific effect of ATP on "myosin" discovered by Engelhardt, Ljubimova and Meitina (1941).

If actomyosin is stored in dehydrated condition, links are developed which are not quantitatively split by ATP. Such links develop especially fast in contracted, thus discharged, actomyosin. Their development is favored by parallel setting.

These observations on elasticity and its *post mortem* changes are in agreement with previous findings of Th. Erdős (1943), corroborated and extended by Bate-Smith and Bendall (1947).

The weight of the autones

The ΔF curve (Fig. 1) shows the free-energy change of the single autones at any given temperature. If the weight and myosin content (8 per cent) of the muscle are known and the ΔF curve and measurements of the total work are accepted, the weight of the single autones can be found by simple numeric calculation.

If, for instance, at a given temperature the ΔF curve indicates an expenditure of 5500 calories per unit, and our piece of muscle performed 0.0055 calories' worth of work and contained 35 mg. of myosin, then the weight of the myosin-unit which has spent 5500 calories would have been 35,000 gm., and this would be the unit weight of myosin contained in one autone. As has been shown (Figs. 6 and 9), isotonic measurements and isometric measurements up to the break indicated a unit weight of 35,000 gm. for myosin. Above the break, the isometric experiments do not yield correct values. At the temperature at which the ΔF curve cuts the 11,000 calories level (53° C. in the rabbit and 47° C. in the frog), the unit weight calculated from the work done by the isometric method must be the double of 35,000 gm. In a series of experiments the unit weight of myosin was calculated from the work done by the sartorius as measured by the isometric method at 47° C. The freshly isolated sartorius was in these experiments connected to the isometric lever and dipped into Ringer of 47° C. The results are given in Table 1.

TABLE 1

	74,000
	66,000
	74,000
	74,000
	69,000
	70,000
	72,000
Average	72,000

This calculated unit weight of 35,000 gm. is based on the current myosin estimations. Should muscle be found to contain more myosin than 8 per cent, this would mean that the unit weight of myosin taking part in the building of one autone is correspondingly higher. There are indications suggesting that the psoas actually contains more myosin than 8 per cent. Moreover, if there is a loss of free energy, this also entails a bigger unit weight. So 35,000 gm. is rather an order of magnitude and the lower limit than the absolute value, which might be equally well 70,000 gm. H. B. Bull (1946) arrived along different lines at a unit weight of 40,000 gm.

CONSIDERATIONS

It may be asked how far the observations made on the psoas of the rabbit, a specific case, reflect a more general behavior. There are different kinds of muscle with widely different functions and structure. As reported before, the contractile matter of all these different muscles seems to be similar, and actin and myosin prepared from cross-striated, smooth, or heart muscle, or even myomas, can be interchanged to form actomyosin which contracts on addition of ATP. Even clam muscle shows similar reactions (A. Lajta, 1947).

There are indications suggesting that the regularities observed are not limited to the contractile matter. If the muscle is minced soon after death and suspended in an alkaline 0.6 M KCl solution, a sticky extract is obtained which owes its high viscosity to the dissolved fibrous structural protein, actomyosin. The hydration and dissolution of this protein is not merely a result of its interaction with the salt-solution. The ATP present has a decisive influence, and if we store the minced muscle for a few hours prior to extraction, giving time for the decomposition of ATP, the subsequent extraction will yield an extract of low viscosity containing no actomyosin. Addition of ATP will restore conditions found in fresh muscle.

As shown by Lajta (unpublished), kidney and other tissues behave in an analogous way. The fresh mince, if suspended in the alkaline salt solution, yields a sticky, highly viscous extract, and the strong double refraction of flow reveals the presence of dissolved fibrous structural proteins. If, however, the mince is incubated, the subsequent extraction yields a fluid of low viscosity containing no fibrous proteins. During the incubation the labile phosphate present disappears. Contrary to muscle, however, the original condition cannot be restored by the addition of ATP or a fresh boiled juice. The labile phosphate, the disappearance of which seems to be connected with this change, is found to be linked to nucleic acid present in the protein. The nucleic acid, prepared from fresh kidney, shows a high content of labile phosphate.

This behavior is completely analogous to that found in muscle, with the difference that instead of a single nucleotide, ATP, in kidney and other parenchymatous organs we find nucleotides united to long chains, to nucleic acid. In muscle, such long chains would interfere with motility.

The close analogy with muscle suggests that in other organs, too, the protein is built of small functional units, each correlated to a nucleotide which governs its physical state and enables the system to develop the two different states, the high-energy, charged, hydrated state corresponding to rest, and the low-energy level corresponding to activity.

SUMMARY

Material and methods of measurement of physical properties of muscle were discussed.

Heat contracture, total work of muscle, and thermodynamic reversibility were studied and found to be in agreement with earlier assumptions.

Elastic properties of muscle and their relation to ATP were studied.

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ON THE RELATION BETWEEN TENSION AND ATP IN CROSS-STRIATED MUSCLE

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According to the theory outlined in the preceding paper, the contractile matter of muscle is built of functional units containing myosin, actin, and ATP. Since muscle contains no free ATP, it can be expected that if the ATP concentration of muscle decreases, the number of contractile units decreases proportionately. The ATP concentration of muscle decreases after the death of the animal (Th. Erdős, 1943). The object of the present research was to see whether the ATP content and the tension developed by muscle decrease proportionately. Such a parallelism would support the theory outlined, while a lack of parallelism would plead against it. For this reason, we measured, simultaneously, the tension developed by the muscle and the ATP concentration at various intervals after the death of the animal.

The material used was the musculus psoas of the rabbit. At various intervals after the death of the animal, strips of this muscle were cut out and frozen at once. The tension developed on thawing was measured.

The methods hitherto used for the estimation of ATP were found to be unsatisfactory for the following reason: we possess no direct method for the estimation of ATP. When this substance has to be estimated, extracts of the tissue are subjected to limited acid-hydrolysis, and the quantity of ATP is calculated from the quantity of labile phosphate liberated. Muscle contains *in vivo* a not inconsiderable amount of free phosphate. As the ATP is gradually decomposed *post mortem*, the amount of hydrolyzable phosphate decreases while the amount of free phosphate increases, and thus a slight error in the phosphate estimation makes the results of the ATP estimation doubtful. A new method of phosphate estimation had to be constructed in which the free phosphate did not interfere with the estimation of the ATP.

In the first part of this paper this method will be described. In the second part, the results obtained by this method will be given.

METHOD OF PHOSPHATE ESTIMATION

The method is based on the ready solubility of phosphomolybdic acid in iso-butyl alcohol, described by Berenblum and Chain (1938), and on the yellow color with which the acid dissolves in this reagent. The muscle was extracted with trichloroacetic acid. Ammonium molybdate was added to the solution. The free phosphate present combined with the molybdate and was shaken out with a mixture

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² Sponsored by the American Heart Association.

of iso-butyl alcohol and ethyl ether. Then the fluid was hydrolyzed and the free phosphate shaken out with iso-butyl alcohol and estimated colorimetrically.

After the trichloroacetic acid extract of the muscle is shaken out with butyl alcohol ether, it still contains a small quantity of phosphate. This quantity can be estimated and taken into account. If the extract is shaken out a second time with alcohol ether, no phosphate is left, and no correction has to be made on the final readings. In the present paper the former method was used.³

Muscle extract contains substances which, after boiling with HCl, yield products which interfere with the development of the yellow color. These substances are eliminated by the alcohol ether, since they are of lipoidic nature.

The detailed description of the procedure is as follows: the rabbit (2-3 kg.) was decapitated, eviscerated, the side walls of the abdomen cut off and the psoas exposed. Two thin strips of the psoas were taken out, provided with ligatures, fixed and frozen with dry ice at their resting length, as described in the preceding paper. These strips were used for estimating the maximum tension developed by the muscle on thawing at 15° C. Simultaneously, a somewhat thicker strip of about one gram weight was cut out from the same region, weighed and frozen. This strip served as material for the ATP estimation. The remainder of the muscle, left *in situ*, was covered with cotton wool wetted with Ringer. The procedure was repeated once every hour. First the right and then the left psoas was used. Such samples were taken until the muscle showed no elasticity and no contractility after thawing. One hour later a last sample was taken.

Extraction: 25 ml. of 10 per cent trichloroacetic acid was pipetted into a mortar which was pre-cooled to - 20° C. The fluid solidified to a brei. The muscle, after having been weighed, was placed into the brei in frozen condition and ground to a fine suspension. On thawing, the suspension was transferred into a centrifuge tube and spun. The clear fluid was poured into a 50 ml. graduated measuring cylinder provided with a ground glass stopper. The volume was noted; then for every 10 ml., 1 ml. of 10 per cent ammonium molybdate solution was added and the fluid mixed. Then 1 ml. of iso-butyl alcohol was added for every 4 ml. of the fluid, and 4 ml. of ether added for every ml. of butyl alcohol used. The fluid was strongly shaken for twenty-five seconds and allowed to separate. If there was no ready separation of the two phases, the fluid was centrifuged. Then the ether butyl alcohol mixture was sucked off through a capillary glass tube. A few ml. of ether were added without shaking in order to wash off the remaining alcohol ether. The volume of the fluid was noted. If, after the shaking with alcohol ether, a heavy precipitate was formed, this was separated by centrifugation. The fluid was divided into samples, each of which corresponded to 100 mg. of muscle, and pipetted into test tubes. Out of nine samples four were put aside. To five samples, 1/10 parts of concentrated HCl (approximately 10 N) was added and the tubes placed into the boiling water-bath for seven minutes and then rapidly cooled. To the unboiled samples, the same amount of HCl was added. To all tubes one drop of 0.1 per cent potassium permanganate was added which stained the fluid a rose color. This color persisted for about half a minute. This was done in order to oxidize any reducing agent present which would reduce the phosphomolybdate. Then 10 per cent ammonium

³ If for any reason the quantity of free phosphate present in the muscle extract had to be known, this could be estimated colorimetrically in the combined alcohol ether extracts.

molybdate was added to the unboiled tubes, and 5 ml. iso-butyl alcohol to all samples. The butyl alcohol used here was shaken out previously with water. (This is necessary in order to prevent the butyl alcohol from taking up water later.) The fluid was shaken strongly for five seconds, the opening of the tube being closed by the thumb covered by a rubber glove. After the two phases separated, the watery phase at the bottom was sucked off by means of a thin glass tube, connected to the vacuum by a thin rubber tube which was pinched tight while the tip of the tube was passing the alcohol. Then the alcohol was poured over into the colorimeter tubes which were marked at their 5 ml. volume. Usually the volume of butyl alcohol is less than 5 ml. It was filled up to 5 ml. with butyl alcohol which was used to rinse the tubes that contained the extract previously. Then to every tube 1 ml. of ethyl alcohol was added and the color estimated in the Klett-Sumner colorimeter with the S 42 blue light filter (400–460 $m\mu$).

As a standard, a solution of KH_2PO_4 was used, containing 0.01 mg. per ml. Samples of 1, 2 and 3 ml. of this fluid were filled up with water to 4 ml., 0.5 ml. cc. HCl and 0.5 ml. of 10 per cent molybdate were added; then the fluid was shaken out with 5 ml. butyl alcohol which was treated as described above.

EXPERIMENTAL RESULTS

Before embarking on the problem proper, a few minor points had to be cleared up. First, is the method of P estimation reliable, and is the distribution of ATP in the psoas homogeneous?

A rabbit was killed and six samples of 1 g. were taken from different parts of the two psoas muscles. In Table I the actual colorimeter readings are reproduced. The six upper columns related to the unhydrolyzed extract are thus the zero values. The corresponding readings of the hydrolyzed samples are reproduced in the lower columns.

As can be seen, the readings are very uniform. The one value in the fourth column, marked with an asterisk, is evidently due to some rough mistake and has to be discounted. The other single values do not differ from the average by more than five per cent. The average of the 0 value was subtracted from the average of the hydrolyzed product. From this the ATP was calculated. The standard with

TABLE I

	40	38	30	29	24	29
	39	35	30	69*	24	28
	39	37	30	29	24	28
	202	194	190	204	198	204
	200	189	200	200	194	202
	204	194	198	206	196	202
	190	196	189	200	200	198
	196	199	187	204	208	194
	195	186	198	214	195	195
Average	198	194	194	205	198	199
ATP	3.55	3.50	3.47	3.70	3.68	3.60 mg. per gm.

0.02 mg. phosphate gave a reading of 80. The quantity of P found was multiplied by 8.4 to give the ATP, which is noted in the last horizontal line. This shows the ATP content of the psoas to be very uniform, 3.6 mg. ATP per gm.

According to the literature, muscle contains 2-2.5 mg. ATP per gm., thus considerably less than psoas. This difference is probably due to the shielded position of the psoas and the consequent poverty of connective material. In order to elucidate this point, samples of different muscles of a freshly killed rabbit were taken and subjected to analysis. Results are reproduced in Table II.

TABLE II

Psoas	3.55 (mg. ATP per gm.)
Deep muscles of the back	3.04
Big adductor muscle	2.56
Musculus gracilis	2.10
Superficial muscle of the back	2.10
Smaller muscles from the gluteal region	1.96

These values show that the more superficial the position and the richer the connective tissue, the lower the ATP content. The muscles of the whole animal would give an average of about 2.5 mg. ATP per gm.

The third question which had to be cleared up was whether the ATP content of the psoas decreases uniformly in all its parts after the death of the animal. Preliminary experiments have shown that the rate of disappearance of ATP *post mortem* depends on the temperature and the oxygen supply. If the muscle is cut into thin strips which are exposed to air, the disappearance becomes much slower. While the ATP in the muscle left *in situ* may disappear within three to four hours, muscle strips exposed to air may contain ATP and thus remain contractile at room temperature even twenty-four hours after the death of the animal. Experiment also showed that in the muscle left *in situ*, the ATP disappeared faster in the deeper-lying dorsal than in the superficial ventral part.

Experiment: The rabbit was killed, the psoas exposed as usual and covered with wet cotton wool. Three hours later, five strips, weighing approximately 1 gm., were cut out and analyzed for ATP. One of the strips was taken from the lateral edge of the muscle, two from the ventral surface, two from the deeper-lying dorsal surface. ATP (mg.) per gm:

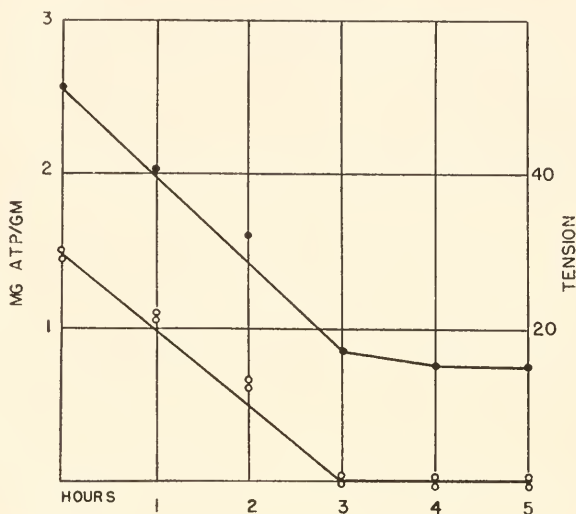
edge:	2.53	
ventral:	2.77	2.77
dorsal:	1.80	1.95

If the ATP content of muscle and its tension are to be measured simultaneously, it is essential that strips from the same region be used for both measurements. Even with this precaution, considerable scattering of results can be expected.

The relation between ATP content and tension was studied in eight experiments. The following example may be cited (Fig. 1): Samples of muscle were taken every hour after the death of the animal. The ATP content in milligrams per gram of muscle is marked in the curve by points. They relate to the left ordinate.

The tension developed is marked with circles and refers to the right-hand side ordinate. (The scale of this ordinate is arbitrary and is chosen in such a way that the numbers, if multiplied by 100, give the total working capacity in calories calculated for 35,000 gm. myosin by the formula: $\frac{1}{2}$ tension $\times$ length $\times$ 0.000023.)

As the curve shows, tension and ATP content run parallel. At the end of the third hour the muscle develops no more tension and does not contract on thawing, and is found to be completely inelastic. At this point, the ATP curve shows a break and becomes roughly parallel to the abscissa.



As the curve shows, the muscle at this point still contains a not inconsiderable amount of labile phosphate. Whether this hydrolyzable ATP is derived from ATP or some other source (ADP?) cannot be stated at present. If this hydrolyzable P is derived from ATP, this ATP must be in some way different from the rest, because it is no longer split by the muscle (or is split only exceedingly slowly) and has no influence on contractility and elasticity. This "residual" hydrolyzable phosphate was found in approximately the same proportion in all experiments.

The second point, equally borne out by the other experiments, is that the decrease of ATP concentration is linear: the rate of its disappearance is independent of its concentration. The most likely interpretation of this rather unexpected fact is that the splitting of ATP depends on some change in the contractile matter. As has been shown by A. Biro and A. E. Szent-Györgyi (unpublished), myosin is enzymatically active in its contracted condition only.

In two out of the eight experiments, the ATP concentration did not fall at all during the first hour after death. This can be explained by the presence of creatine-phosphate which rephosphorylates the ADP formed.

All experiments gave similar results. In most of them the scattering was stronger than in the quoted example. Nevertheless, all experiments bore out the close parallelism between tension developed and the quantity of ATP present.

SUMMARY

A new colorimetric method of ATP estimation is described. In the psoas of the rabbit the *post mortem* decomposition of ATP and the loss of contractility are parallel.

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EARLY STAGES IN THE LIFE HISTORY OF THE COMMON MARINE SHRIMP, *PENAEUS SETIFERUS* (LINNAEUS)

WILLIAM W. ANDERSON,¹ JOSEPH E. KING,² AND MILTON J. LINDNER³

The purpose of this report is to assemble information concerning the early stages in the life history of the shrimp, *Penaeus setiferus* (Linnaeus), which supports the most valuable commercial fishery of the South Atlantic and Gulf of Mexico regions of the United States.

P. setiferus is an organism of high reproductive potential. A count made by the authors on the ripe ovaries of a female, 172 mm. total length with spermatophore attached, revealed a total of approximately 860,000 eggs. Burkenroad (1934) states that the ovary of a large shrimp may contain 500,000 eggs. Heldt (1938) counted about 800,000 eggs in the ovaries of *P. trisulcatus*, a European species of similar size and closely related to *P. setiferus*. It may be expected, therefore, that a female will produce from 500,000 to 1,000,000 eggs in a single spawning.

DESCRIPTION OF THE EGG

The size of the ripe ovarian egg has been given by Weymouth, Lindner and Anderson (1933) as ranging from 0.25 to 0.33 mm. in diameter with an average of 0.277 mm. Burkenroad (1934) believed the egg to be about two-thirds this size, or about 0.185 mm. Later he changed this estimate to "—about 0.25 mm. or less," (Burkenroad, 1939). Pearson (1935), through the use of a plankton net, secured nauplius-bearing eggs varying in diameter from 0.38 to 0.42 mm. Later he reports (Pearson, 1939) that the diameter of twenty-five live eggs, also secured with a plankton net, uniformly measured 0.28 mm. Gutsell (1936) obtained measurements ranging from 0.192 to 0.300 mm. on ripe eggs from a female with spermatophore attached. He found that fresh oocytes dissected out in sea water were about 0.30 by 0.36 mm. Much of the variation in the data cited may be due to varying age of the eggs and varying manner in which they were handled (some were measured fresh, others after fixation).

According to Pearson (1939) "the egg of *P. setiferus* is demersal and sinks promptly in still sea water." It is "non-adhesive and spherical" and "possesses a thin transparent membrane, or chorion, that in living and preserved eggs shows a characteristic purplish-blue color in reflected light under the microscope."

LARVAL DEVELOPMENT

Our knowledge of the larval development of *P. setiferus* depends largely on the work of Pearson (1939). To quote from the summary of his paper: "The larval development of *Penaeus setiferus*, the common commercial southern shrimp, consists of ten distinct stages excluding the demersal spherical egg. These stages

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³ Aquatic Biologist, Office of Foreign Activities, U. S. Fish & Wildlife Service.

are made up of five forms generally included under the name of nauplius, three forms included under the name of protozoa, and two forms included under the name of mysis." In addition to these ten larval forms, Pearson describes two post-larval stages which precede the true adult form.

In brief, the larval development of *P. setiferus* requires from two to three weeks. Some twenty to twenty-four hours after the egg is spawned the nauplius breaks the chorionic membrane and emerges. Its ovoid body, of 0.30 to 0.34 mm. in length, bears a single, simple eye and three pairs of oar-like appendages which are to become eventually the first and second antennae and the mandibles. Although this minute organism is to a great extent at the mercy of the prevailing currents, it is capable of some movement. In the next twenty-four to thirty-six hours the nauplius undergoes five successive molts to become a protozoa of approximately 1 mm. in length. It now has seven pairs of appendages, a pair of sessile compound eyes in addition to the ocellus, and a complete alimentary tract consisting of mouth, esophagus, stomach, intestine, and anus. Prior to this stage the food of the nauplius has been the yolk material carried over from the egg. This food supply is now exhausted and henceforth the protozoa must capture its own food if it is to survive. This transitional period is without doubt a critical one in the animal's life history.

The third protozoa stage is followed by the first mysis, which is about 3.5 mm. in length, possesses fourteen pairs of functional appendages, and on the abdomen five pairs of buds which will soon become the pleopods. In the second mysis the pleopods are well developed, and rudimentary gills have made their appearance on the thoracic somites. With the succeeding molt the organism ends its larval phase and assumes the general proportions of a miniature adult. At the end of two post-larval stages and fifteen to twenty days after hatching, the young shrimp is only 5 to 6 mm. in length and is still planktonic. During this period of early development, the young shrimp have moved from the saline offshore spawning area to the brackish inside marshes, bays, and estuaries (Weymouth, Lindner and Anderson, 1933). Upon reaching these "nursery grounds" they adopt for the first time, it is believed, a benthic existence.

The factors responsible for this inshore movement of larval and post-larval *P. setiferus* have not been determined. We believe, however, that for the young to reach the nursery grounds they must encounter a favorable incoming current. While capable of some movement, and perhaps responsive to a salinity gradient, they would certainly be quite helpless against out-going currents. *P. setiferus* has a long spawning season, which in Louisiana extends from March to September; consequently, at intervals during this period the young are bound to encounter favorable conditions for their inshore migration.

Although spawning usually takes place offshore, schools of adult shrimp have been known to approach the coast and spawn close to inlets. When such a spawning occurs, the eggs may be swept through the passes on incoming currents, and the larvae may reach the nursery grounds within a few hours.

YOUNG SHRIMP

As stated above and previously reported by Weymouth, Lindner, and Anderson (1933), young shrimp approximately 7 mm. in length are found during the early

spring months in the brackish inside areas which serve as their nursery grounds for the next four to eight weeks of their existence. This habitat is a rich feeding ground characterized by shallow water, muddy bottoms, rather widely fluctuating seasonal temperatures, and moderate to low salinity. Numerous seine and frame-net collections in these areas have yielded quantities of small shrimp 7 to 10 mm. in length; whereas frequent hauls with the same gear during the same period along the ocean and Gulf beaches have failed to yield any *P. setiferus* of this size, although other species of shrimp were found.

As the young grow, they move from the shallow waters of marsh, bayou, and lagoon into the deeper creeks, rivers, and bays, making their first appearance on the inside fishing grounds when about 50 mm. long. In Louisiana the lower limit of the size distributions obtained from operations with commercial gear in the fishery was 43 mm., in Georgia 58 mm., and in Texas 63 mm.

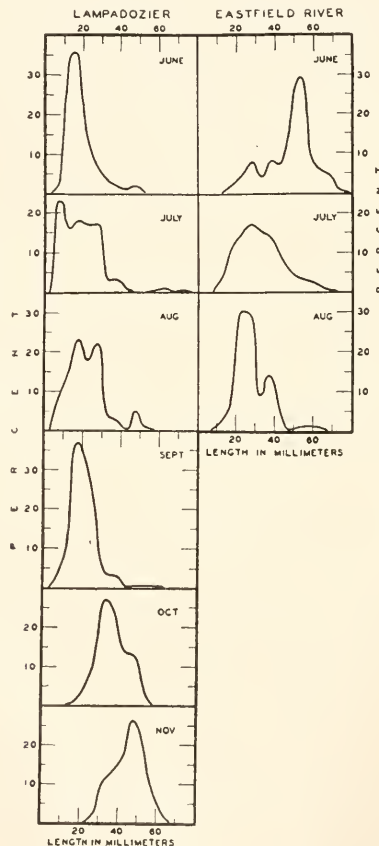


FIGURE 1. Size distribution of young shrimp (*Penaeus setiferus*) seined from the nursery ground areas of Lampadozier and Eastfield River, Georgia. Males and females combined. For the Lampadozier series the curves are based on 200 shrimp in June, 200 in July, 100 in August, 200 in September, 100 in October, and 92 in November. The Eastfield River series are based on 100 shrimp in June, 400 in July, and 100 in August.

To illustrate the population on a typical nursery ground area, the length frequency distributions of small shrimp from seining and frame-net operations in two localities in Georgia are shown in Figure 1. The Lampadozier series, which covers a period from June to November, was obtained entirely by seining in one particular locality in a section representing the inner reaches of the nursery grounds. The young shrimp of the Eastfield River series were somewhat larger in size; they represent collections (covering a period from June to August) taken with both seines and frame-nets in an area midway between the upper nursery grounds (represented by the Lampadozier series) and the lower bays or sounds. The apparent reverse order in the sizes of shrimp in the Eastfield River series is due, it is believed, to the exodus late in June of the larger shrimp, a product of an early spawning, and the entrance in great abundance into the River in July and August of the young from the peak spawning period of May and June.

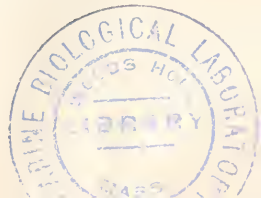
In the Lampadozier section during June and July the average length of the shrimp was 18 mm. with a range from 8 to 48 mm., although in July a few scattered longer individuals were obtained. In August the average length had increased to 23 mm. with a range from 8 to 53 mm. During September the average length was maintained at 23 mm. with the bulk of the population ranging between 8 and 38 mm., although scattered individuals up to 78 mm. in length were secured. By October the average length had increased to 38 mm. with a range from 18 to 53 mm. During November the average length rose to 48 mm. with a range of 28 to 63 mm.

From the Lampadozier data, the increase in the lower limits of the length frequency distribution from 8 mm. in September to 18 mm. in October and to 28 mm. by November, indicates that after September no new recruits were appearing on the nursery grounds. September marks the end of the spawning season in Georgia (Anderson, Lindner and King, 1948).

RELATIONSHIP OF NURSERY GROUNDS TO COMMERCIAL CATCH

The distribution of the shrimp fishery in itself obviously indicates that passes and the adjacent inland waters are of prime importance to the species. Louisiana, which has a combination of more passes and a vastly larger inland water area landward of these passes than any other state, produces about two-thirds of the shrimp caught each year throughout the entire South Atlantic and Gulf region. Likewise, Georgia and South Carolina, whose shorelines have the most numerous passes and favorable inside waters on the South Atlantic Coast, develop the greatest numbers of shrimp in that section. As a consequence, we conclude that the number of openings to the outside waters and the extent of favorable nursery grounds are two of the major physical factors influencing the production of shrimp in the various sections of the fishery.

In addition to the number of passes and the area of nursery grounds, a coastal or nearby offshore area of relatively shallow water, high salinity, and mud or clay bottom also seems to be a requisite. The Florida peninsula between Fort Pierce on the east coast around almost to St. Marks on the west coast lacks this and likewise lacks shrimp. It is not yet known whether this factor is a requirement of adults or larvae or of both.



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MITOCHONDRIAL ARRANGEMENT IN ALVEOLAR EPICYTES AND FOAM CELLS OF MOUSE LUNGS, PARTICULARLY AS INDUCED BY THE VACUOLOIDS¹

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INTRODUCTION

The problem of the causation of the design taken collectively by the mitochondria in a living or a fixed and stained cell has interested cytologists for many years. For instance, Lewis and Lewis (1915, p. 352), in studying the living cells of tissue cultures, ask: "What is it that governs the arrangement of the mitochondria? Is it the shape of the cell, the influence of the central body or of the nucleus, the internal structure of the cytoplasm, or do the metabolic activities of the cell govern the size, shape and arrangement of the mitochondria?" Full answers to these important questions have not yet been forthcoming, and the problem is a complicated one. No doubt each of the factors mentioned plays a part.

It seems clear, too, that the physical influence of mechanically inert bodies in the cytoplasm is a formative factor which may be predominant, for mitochondria occupy the general cytoplasm and not the special masses, living or dead, which may find a place in it; and hence anything which molds this general cytoplasm will incidentally establish the morphological pattern of the mitochondria in it. Thus Cowdry (1914) found that the mitochondria of large spinal ganglion cells occurred between the flakes of Nissl substance, and Thurlow (1917) observed that in nerve cells of the cranial nuclei the mitochondria avoided the canalicular apparatus. Similarly the Lewises (1915) showed, in their Figure 26, the mitochondria arranged in a network around the fat droplets of a tissue culture cell. Foreign body inclusions act likewise. This simple mechanical influence on mitochondrial arrangement is herein shown to be exerted by vacuoloids in the pulmonic alveolar epicytes and foam cells to such a degree that the ensuing picture is outstanding and characteristic.

Alveolar epicytes are the residual epithelial cells in the pulmonary alveolar walls (Macklin, 1946). They are also called "septal cells," "niche cells" and other names. Although in the marginal alveolar bases (Macklin, 1945) and other places they have but one air face and rest upon connective tissue, in the interalveolar partitions they frequently have two air faces. These are often of unequal size, the larger overlying the head of the cell and the smaller the foot. In silverwashed material the head and foot are each encircled by a line of silverized material which is part of the silver lineation of the alveolar walls and bronchioles (Macklin, 1938). At alveolar wall intersections the epicytes, as herein discussed, not seldom have three air faces (Fig. 5). This trifaciality is like that found in certain dust cells of mouse lungs

¹ A grant in aid of this investigation by the National Research Council of Canada is gratefully acknowledged.

treated with ammoniacal silver solution (Macklin, 1948). Epicytes may assume a phagocytic rôle (Macklin, 1946). Other functions have been ascribed to them (Hayek, 1942; Sjöstrand and Sjöstrand, 1938). That they may, on occasion, become malignant, so initiating a primary cancer of the lung, has been admitted as a possibility (Macklin, 1938). Their most prominent and characteristic feature is an array of vacuoloids which occupies much if not most of the cytoplasm (Macklin, 1947a). These are clear, round, discrete, non-lipoidal bodies averaging $0.5\ \mu$ to $0.75\ \mu$ in diameter, which do not take stains (Brodersen, 1933). They are relatively stable, indenting the nucleus in fixed and stained sections.

Alveolar foam cells of mammalian lungs have been described with special reference to their vacuoloids (Macklin, 1947b). They are found in ordinary histological sections, and some of them may be recovered by what has been termed the "gash-irrigation" technic, in which the fresh, collapsed lung is incised through a drop of physiological saline solution and the preparation inverted over a glass slide on which is received the emerging fluid carrying loose cells from the peripheral alveoli. This fluid is then spread and stained as for blood. Foam cells are regarded as originating from epicytes and possibly also from the diversifying epithelium of the bronchioles at the marginal zone adjoining the alveolar ducts, and as being developmental brothers of the dust cells. Difficulty may be encountered in distinguishing the smaller foam cells from well developed epicytes, in sections. The alveolar foam cells are thus of entodermal origin, and are not to be confused with the mesodermal "foam cells" of the pathological literature. As the name implies, they have a foamy appearance, the numerous vacuoloids accounting for the clear spots, which in sections often misleading seem to be merged. In dry smears the vacuoloid diameter may reach $1.5\ \mu$.

There is no reason to suspect that the mitochondrial arrangements hereinafter described are peculiar to the alveolar epicytes and foam cells of the mouse. They are probably to be found in these cells throughout the mammalian class at least.

MATERIAL AND METHOD

This short study is upon albino mouse lungs freed from as much blood as possible by hemorrhage and moderately distended by the prompt intratracheal injection of Regaud's fixing fluid. Paraffin sections, stained by Bensley's adaptation of the Altmann technic (Cowdry, 1943), reveal the mitochondria in brilliant ruby-red. Most of the mitochondria in epicytes and foam cells are of round or oval form. These are seen in all parts of the general cytoplasm. The ovals grade into short thick rods with rounded ends. Filaments, often beaded, occur. Mitochondria differ in size, the largest being conspicuous while the smallest are seen with difficulty. It is possible that the degree of differentiation with picric acid has something to do with the optical impression of size, which seems less in over- than in under-differentiated cases; but this factor can hardly be in operation when mitochondria in the same cell are being considered, for these have presumably been subjected to uniform technical action. It is the impression that the mitochondrial content of the well developed epicytes is more conspicuous than that of the simpler epicytes on the one hand and the larger foam cells on the other. These mature foam cells are lighter in color, lacking the marked rosy hue typical of the mature epicytes, and in them the mitochondria appear generally smaller and more weakly staining than in the smaller foam cells and epicytes, and are predominantly of coccoid form. Thus

there is considerable variation in the relative prominence of the mitochondrial picture in the various cells examined.

PERIVACUOLOIDAL GROUPING OF MITOCHONDRIA

The design of groups of mitochondria in typical epicytes and foam cells, as seen in well stained thin sections, is dominated by the presence and spacial disposition of the vacuoloids and appears characteristically as a round-meshed sieve with circles of mitochondria, the perivacuoloidal clusters, bounding these clear spheroidal bodies. Mitochondria are never admitted to the interiors of the vacuoloids.

In Figure 2 is shown one of the smaller bifacial epicytes in which the structure is relatively simple, the vacuoloids being in only one layer. Perivacuoloidal groups *p v g* surround these bodies. Those mitochondria which lie just beneath the cell membrane may take part as well in the formation of the inframembranal group *i m g*, and similarly those which are immediately around the nucleus participate as well in the makeup of the perinuclear group *p n g*. This is a good example of an epicyte which goes right through the alveolar wall, and it shows a larger end, the head, well filled with vacuoloids, and a comparatively narrow shank or trunk with a small foot.

Where greater numbers of vacuoloids occur, the appearance is more complex. In the epicyte seen in Figure 3, there are many perivacuoloidal groups, mainly of ovals and spheroids. This cell appears lodged in a crotch of alveolar walls. It has a free air-face above, another to the left and a third to the right, represented in the small foot at the end of the narrow shank which is within a space of the alveolar wall like a pore. Above are rods just beneath and parallel with the free face *i m g*.

In the larger epicytes and foam cells the vacuoloids are in several layers, and hence the perivacuoloidal mitochondria comprise relatively much more of the total content. Figure 7 gives a good impression of this sievelike pattern. It is from a very thin slice of a foam cell at the side of the nucleus. The inframembranal layer is incomplete. In thick sections one can focus up and down through the numerous vacuoloids and find impressive numbers of mitochondria in the cytoplasm around them.

Figures 8, 9 and 10 show this perivacuoloidal arrangement in other foam cells. Under the oil immersion lens the overall picture is uniquely beautiful, and once seen is not forgotten. It is like chains of brilliant rubies festooned about large luminous pearls. Photographs at best give an inadequate representation. The numerous and often large mitochondria are mainly round or oval, and most of them are about the vacuoloids, with an incomplete layer under the cell membrane and another over the nuclear membrane.

Sometimes epicytes which appear to be underdeveloped are found, showing relatively few vacuoloids or mitochondria. In Figure 1, for instance, there is a single row of vacuoloids present only on the air surface. But one mitochondrion appears on the side next to the air, and it is between two vacuoloids. This is an example of a very simple distribution of mitochondria. They are massed above and to the right, in the cytoplasm of the surface which rests on connective tissue.

It does not appear that the mitochondria are attracted by the vacuoloids, but rather that they occupy inertly the available space around them. By no means all of the vacuoloidal surface is contiguous to mitochondria. Most of the mitochondria around the vacuoloids are of the spheroid or ovoid type; but there

PLATE I

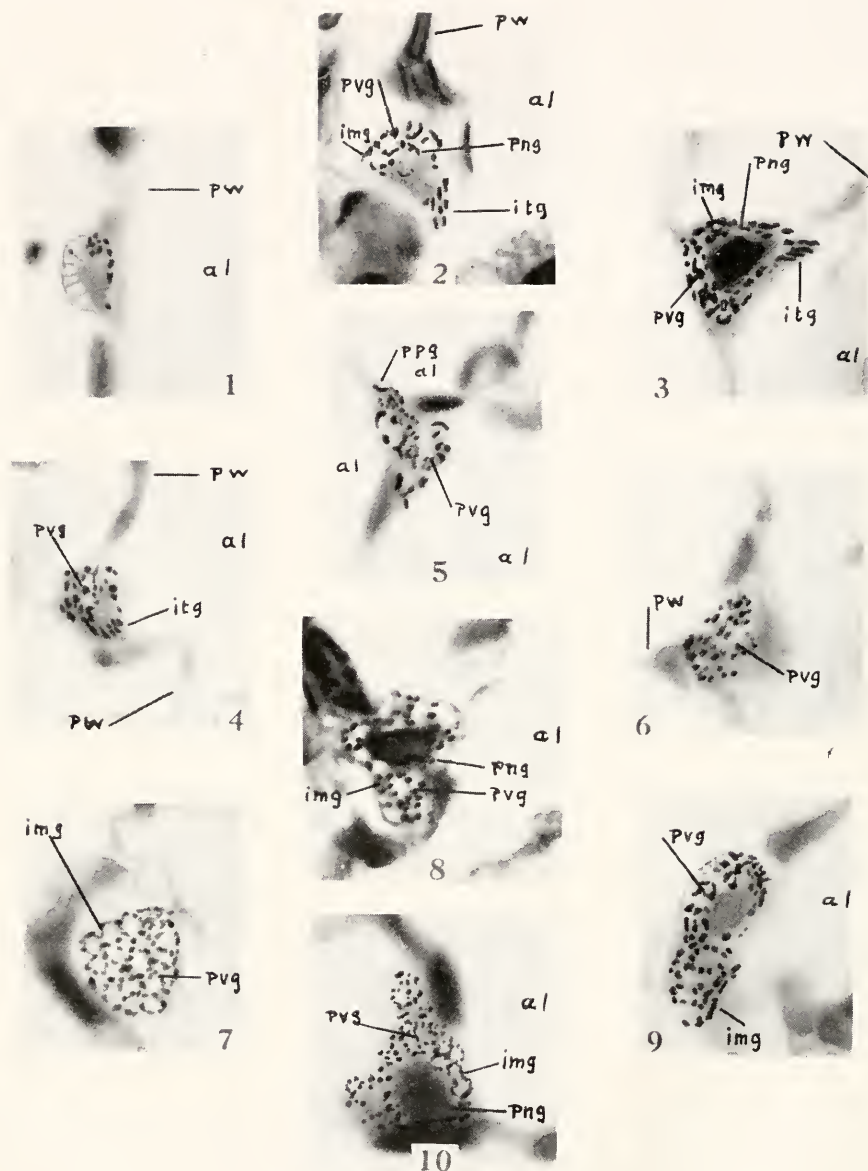


PLATE I

The ten figures are photomicrographs at 1900 diameters made with a Bausch and Lomb 1.9 mm. 1.32 N.A. oil immersion fluorite system objective and 10 $\times$ ocular from 3 μ Altmann sections of mouse lungs prepared as described. On the prints the mitochondria were intensified with India ink applied with a fine pen on consultation of the original cell under the oil immersion lens. The first six figures are regarded as epicytes and the remainder as foam cells. Details of mitochondrial arrangement are given in the text.

are many short rods and these are found with the long axis lying tangentially to the vacuoloidal surface. There is no reason to suggest that mitochondria are in any way concerned in the formation of the vacuoloids or that they are influenced in form, size or any other way by contiguity with the vacuoloids. Experimental swelling and distortion of the vacuoloids is reflected in spreading and attenuation of mitochondrial arrangements around them.

ARRANGEMENTS NOT DETERMINED BY THE VACUOLOIDS

When epicytes are so cut as to show the long axis of the cell approximately parallel with the optical plane, we may see mitochondrial rods of the intratruncal group *itg* lying more or less parallel with one another in the trunk or shank and reaching to the foot (Figs. 2, 3). This region of the epicyte lies within the alveolar wall close to the capillaries. In cross sections of such shanks the now dotlike mitochondria are disposed in a circle. This arrangement suggests the shrunken staves of an empty barrel. When cut at a slant such a group appears as in Figure 4. No reason for this peculiar pattern is apparent.

Mitochondria have been noted in epicytes and foam cells lying close to the cell membrane *img*. As rods and filaments they often lie parallel with this membrane, and sometimes occur in a double row (Fig. 9). Another layer, which may be indefinite and typically composed of shorter forms, has been noted in the perinuclear cytoplasm *png* (Figs. 3, 8, 10). In the edges of the heads of epicytes, where the inframembranal cytoplasm underlying the air surface merges with that adjoining the connective tissue surface, the mitochondria of the peripheral group *ppg* (Fig. 5) in lateral view may have a curious pointed appearance like a pile of sticks, as in the profile of the supports of a North American Indian wigwam, which is difficult to photograph; while other groups simulate the downdrooping branches of the tops of balsam trees. Such clusters contain rounded and oval forms as well as rods. Sometimes mitochondria in these edges are packed in a dense triangular mass. Again, no explanation for these curious formations has been found.

Epicytes on marginal alveolar walls (those which rest on connective tissue) are well endowed with mitochondria. One of these is represented in Figure 6 in the angle between adjoining alveoli. To the left, a group of mitochondria juts into the partition *p ω* separating the upper from the lower alveolar space. Perivacuoloidal formations are seen here as in other epicytes.

Substantially the same representation of mitochondrial arrangement in these cells was obtained after the use of Bensley's acid violet-safranin O (Bensley, 1911; Lillie, 1948, p. 98), and Regaud's modification of the iron hematoxylin method of Heidenhain (Cowdry, 1918), though hitherto, in the author's hands, less brilliantly.

Abbreviations:

<i>al</i>	alveolus,
<i>img</i>	inframembranal group of mitochondria,
<i>itg</i>	intratruncal group,
<i>mω</i>	marginal alveolar wall,
<i>png</i>	perinuclear group,
<i>ppg</i>	peripheral group,
<i>pνg</i>	perivacuoloidal group,
<i>pω</i>	partitional alveolar wall.

SUMMARY

In epicytes and foam cells the combined perivacuoloidal groups of mitochondria present, in thin sections, an outstanding and characteristic lacelike picture based on the disposition and condition of the vacuoloids. Its determination is probably mechanical. Mitochondria are never found within the vacuoloids. An incomplete layer immediately underneath the cell membrane, and another around the nucleus, are found. In the former the rods often lie parallel with the membrane and sometimes in double rows.

In epicytes there is often a distinctive group, mostly of rods, which suggest the outer layer of the fascies, and lie in the long axis of the shank, arranged in a circle around a central area devoid of them. Bizarre angulated and branched arrangements are noted in the peripheries of the heads of epicytes.

In foam cells the mitochondrial content varies, often being abundant and conspicuous, and again, perhaps in older cells, relatively inconspicuous.

ACKNOWLEDGMENT

I wish to thank Mr. Charles E. Jarvis for his excellent work in the preparation of the sections and photomicrographs.

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THE CHARACEAE OF THE WOODS HOLE REGION, MASSACHUSETTS

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INTRODUCTION

It is extremely doubtful that any other comparable area in North America has been subjected to such constant investigation for fresh-water algae over such a long period of time as has the region in the vicinity of Woods Hole, Massachusetts. Specimens which have become widely distributed in herbaria in this country and abroad show that an almost continuous series of collections has been made since the days of Agassiz and the establishment of the original marine biological laboratory on Penikese Island. Among these collections, many specimens of Characeae have been accumulated by generation after generation of students and investigators, until today one finds an abundance of herbarium material available for study. The objectives of the present paper are primarily to present a workable local flora for the Characeae for use of students of phycology and the independent investigator who employs these plants in his research; and secondarily to provide a systematic treatment of the local species based upon a study of available specimens.

During the summer of 1947, the writer undertook a survey of all accessible bodies of fresh and brackish water in the Woods Hole region. Specimens of Characeae were collected from all places where found, and herbarium mounts and preserved material were prepared. Duplicates of representative forms have been distributed to the New York Botanical Garden, to the herbarium of the Marine Biological Laboratory at Woods Hole, and to certain private herbaria.

The Woods Hole region as delimited for the present paper is approximately the same as that covered by Croasdale (1935) with the addition of certain islands. Roughly, the area includes the islands of Nantucket and Martha's Vineyard, the Elizabeth Islands, and the southern tip of Cape Cod. Names of localities and ponds (cf. Table I) follow those used by Croasdale. Ponds in areas not included by that writer and for which the local names are not known to the present writer are assigned local names as indicated on herbarium labels.

The ponds of the region vary greatly as ecological habitats. In general, the isolated inland ponds result from the glacial knob-and-kettle topography, and tend to be slightly to strongly acid. The coastal ponds are largely lagoons which have become partly or totally isolated from the ocean by barrier beaches or bars. These tend to be markedly alkaline, and salinity varies from strictly marine to weakly brackish. These differences are reflected in the quite distinct characean flora of the two types of ponds. Correlations of distribution and ecological variables will be reported elsewhere.

Despite the wide range of aquatic habitats, the characean flora of the Woods Hole region is not great. With the possible exception of *Nitella Morongii* Allen, all the species reported also occur on the mainland. On the contrary, a number of

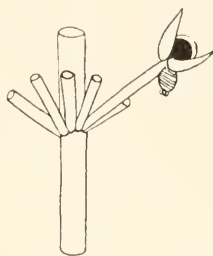
PLATE I



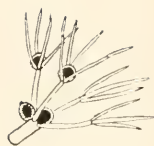
1-A



1-B



1-C



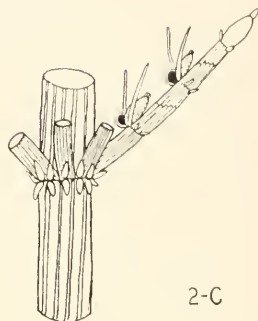
1-D



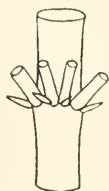
2-A



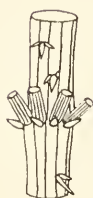
2-B



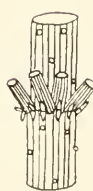
2-C



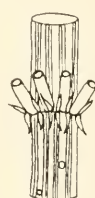
3-A



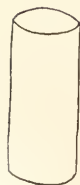
3-B



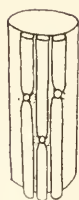
3-C



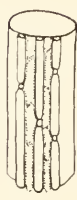
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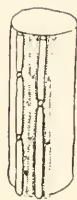
4-A



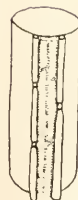
4-B



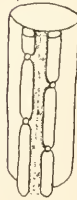
4-C



4-D



4-E



4-F

species are lacking which could well be expected in the region studied, because of their frequent occurrence on the adjacent mainland. Among those noticeable by their absence are *Chara contraria* Kütz., *C. vulgaris* L., *C. sejuncta* Braun, *C. gymnopitys* Braun (= *C. fibrosa* Ag. ex Bruz.),¹ *C. fragilis* Desv. ap. Lois. (= *C. globularis* Thuillier), *N. opaca* (C. Agardh ex Bruz.) C. Ag., *N. acuminata* Braun ex Walzm., *N. capillaris* (Krocke) Groves & B.-W., *N. tenuissima* (Desv.) Kütz., and *TolyPELLA* spp.

In addition to the specimens obtained during this survey, the writer has had the opportunity to inspect material from the region in various herbaria, including the following: Brown University (BRU)—courtesy of Dr. W. H. Snell; Chicago Natural History Museum (F)—courtesy of Dr. F. Drouet; Farlow Reference Library and Herbarium (FH)—courtesy of Dr. W. L. White; Marine Biological Laboratory (MBL); Maria Mitchell Association of Nantucket (MMA)—courtesy of Grace Wyatt; New York Botanical Garden (NY)—courtesy of Dr. D. P. Rogers, Dr. F. J. Seaver, and Rosalie Weikert; University of California (UC)—courtesy of Dr. H. L. Mason and Dr. G. F. Papenfuss; Yale University (Y); and the private herbaria of E. T. Moul (ETM), Dr. M. S. Doty (MSD), and Dr. W. R. Taylor (WRT). The abbreviations indicated for these herbaria and employed throughout the text follow Lanjouw (1939) with the exception of the writer's herbarium (RDW), other private herbaria (ETM, MSD, WRT), and those for which no standard abbreviation is listed (MBL, MMA).

The literature of the Woods Hole Characeae consists of a few scattered papers. Halsted (1879) reported *N. gracilis* (Sm.) Ag. from Nobska Pond and *C. coronata* var. *Schweinitzii* Braun from shallow ponds at Woods Hole. T. F. Allen (1880;

¹ Zaneveld (1940: 153).

PLATE I

Diagrams illustrating terminology and certain morphological details of the Characeae.

FIGURE 1. *Nitella*. Diagnostic features: **1-A**, branchlets in the whorls (once) furcate; two true branches shown. **1-B**, coronula of oogonium with ten cells (6 visible) in two tiers. **1-C**, axial node, without stipulodes; axis ecorticate. **1-D**, one fertile branchlet twice furcate (divided) into four secondary rays at the first furcation and 3-4 tertiary rays (dactyls) at the second furcations; three antheridia shown terminally on rays in whorl of rays of the next order; one oogonium shown lateral at the first branchlet node.

FIGURE 2. *Chara*. Diagnostic features: **2-A**, branchlets in whorls, not furcate; no true branches. **2-B**, coronula of oogonium with five (3 visible) cells in one tier. **2-C**, axial node with two rows of stipulodes (diplostephanous); axis corticated; branchlet not furcate; antheridia below oogonia at branchlet nodes; two elongated bracteoles shown subtending each oogonium; reduced bracts at sterile branchlet node and at fertile nodes abaxial to bracteoles.

FIGURE 3. *Chara*. Terminology of axial nodes: **3-A**, ecorticate, haplostephanous (stipulodes in one row). **3-B**, haplostichous, haplostephanous. **3-C**, diplostichous, diplostephanous (stipulodes in two rows). **3-D**, triplostichous, diplostephanous.

FIGURE 4. *Chara*. Terminology of axial cortication. A few cortical filaments shown on a segment of internode. Primary cortical cells drawn solid, secondary cortical cells dotted. **4-A**, ecorticate (without cortication). **4-B**, haplostichous, secondary cortical cells of each cortical node not elongated. **4-C**, diplostichous, secondary cortical cells about half the length of primary cortical internodal cells, resulting in one row of secondary cells between each row of primary cells. **4-D**, triplostichous, secondary cortical cells about equal in length to the primary cells, resulting in two rows of secondary cortical cells between each row of primary cells. **4-E**, triplostichous, aulacanthous (secondary cortical cells greater in diameter than primary cortical cells). **4-F**, triplostichous, tylacanthous (primary cortical cells greater in diameter than secondary cortical cells).

1882a) reported *C. crinita* f. *leptosperma*; and later (1896) a form near *Nitella minuta* or *N. batrachosperma*, possibly a new species *N. marccana* Allen, from Nantucket; and the description of *N. Morongii* Allen. Maria Owen (1888) listed the following species from Nantucket: *Nitella batrachosperma*, *N. flexilis*, *N. flexilis* var. *subcapitata*, *N. Morongii*, *N. mucronata*, *Chara coronata*, *C. coronata* var. *Schweinitzii*, *C. crinita*, *C. crinita* f. *leptosperma*, *C. fragilis* var. *delicatula*. The most recent work is by Croasdale (1935) who reported *Nitella gracilis* from Wood and Golf Ponds, *N. flexilis* from Cuttyhunk, *N. mucronata* var. *gracillima* from Wood Pond, *Chara canescens* and *C. fragilis* from Chara Pond, and *C. delicatula* from Cuttyhunk. This work included a diagnostic key to the species.

The terminology employed in the present paper follows the majority of recent workers in the field. Because of variations in usage of certain terms in the literature, the writer's usage of each term is defined in the discussions of the class, family and genera. Further, the structures are labeled in Plate I.

The nomenclature is that which, in the writer's opinion, represents the valid name; and where marked deviations from recent monographic treatment exist, reasons for such changes are given. Synonyms listed include only those which occur in recent literature. For more complete synonymies, the reader is referred to Migula (1897), Groves and Bullock-Webster (1920; 1924), Zaneveld (1940), and Wood (1948b) for the genus *Nitella* in North America.

The descriptions and figures, with exception of illustrative diagrams, were taken from specimens collected in the region, and thus reflect only the characteristics of the local forms. All such specimens are in the writer's herbarium (RDW). It should be noted that the description of each species is based entirely on a single specimen. Variations from this plant are to be found in the discussion of variations following each description.

In addition to the persons and institutions indicated above to whom the writer is deeply indebted for the opportunity to study herbarium material, he is especially indebted to Dr. Hannah Croasdale who guided the writer to many collecting sites, and to Dr. M. S. Doty for his encouragement and aid in the completion of the present work. Most sincere gratitude is due G. O. Allen, Esq., of England, Dr. W. R. Taylor, and Dr. M. S. Doty for timely criticisms of the original manuscript.

THE KEY

The following key is intended to be strictly analytical in nature, and employs vegetative characteristics as far as possible. The most convenient characteristics are listed first in each entry. The key disregards the distinctions between genera. A differential synopsis of tribes and genera may be found under "Family Characeae."

- 1a. Main axes uncorticated 2
- 1b. Main axes corticated 6
 - 2a. Branchlet whorls not subtended by stipulodes; branchlets one or more times furcate (forked); antheridia borne terminally on rays; oogonia borne laterally at furcations of branchlets, thus situated below antheridia 3
 - 2b. Branchlet whorls subtended by one series of stipulodes; branchlets not furcate; antheridia borne laterally at branchlet nodes; oogonia above antheridia 5. *Chara Braunii*, p. 193

- 3a. Ultimate rays (dactyls) of branchlets not terminated by a small pointed cell (mucro) 1. *Nitella flexilis*, p. 187
- 3b. Ultimate rays of branchlets terminated by one or more such small cells 4
- 4a. At least some of the dactyls more than two-celled, the tips varying from a single-celled mucro to a tip with two or more such mucros; oogonia geminate (two at a node) to aggregate (more than two at a node), but occasionally solitary; plants large and robust, main axes in mature plants generally exceeding 400 μ in diameter.
2. *Nitella megacarpa*, p. 188
- 4b. Dactyls strictly two-celled including the small terminal mucronate cell; oogonia solitary (one oogonium at a fertile node); plant distinctly small and delicate, main axes generally less than 350 μ in diameter 5
- 5a. Branchlets once or twice furcate; fertile whorls reduced to small heads; oospore membrane appearing roughened, but not reticulate.
3. *Nitella Morongii*, p. 189
- 5b. Branchlets 3 or more times furcate; fertile whorls not reduced to heads; oospore membrane reticulate 4. *Nitella transilis*, p. 191
- 6a. Spines (at least some) on main axes generally in groups of 2-5 (fascicled); cortication haplostichous (number of corticating cells equal to the number of branchlets in an adjacent whorl).
6. *Chara canescens*, p. 196
- 6b. Spines (if present) on main axes not fascicled (very rarely paired). solitary; cortication of the main axis appearing diplostichous to triplostichous (corticating cells 2-3 times as many as branchlets in adjacent whorl) 7
- 7a. Plants monoecious, the oogonia and antheridia on same plant, though by loss of one or other of the sex organs they may appear dioecious.
7. *Chara delicatula*, p. 198
- 7b. Plants dioecious, the oogonia and the antheridia borne on separate plants (thorough inspection of a number of plants must be made before the dioecious condition can be considered proved) 8. *Chara aspera*, p. 199

CLASS CHAROPHYCEAE

More or less bushy, green, submerged, attached, aquatic plants which vary in size from 0.5 cm. to nearly 2 meters in height. Rhizoidal portions hyaline, and ramifying in the soil. Laterals borne in whorls along an erect axis. Certain forms deposit an external incrustation of lime; some become dark brown to black. Both conditions may be uniform or may develop as bands encircling the cells. Habitat strictly fresh water in most species, some species more tolerant of low salinity, and some restricted to brackish water. No marine forms are known.

Vegetative portions fundamentally consisting of a uniaxial filament, the filament exhibiting alternating elongate internodal cells and short nodal cells. The internodal cells merely elongate, but the nodal cells divide three or more times in such a way as to form central nodal cells and a series of peripheral nodal cells. One

or more central nodal cells at each node cut off apical cells, which continue development of filaments (*main axis*). A dominant filament so formed is considered the main axis, others are considered as *branches* (laterals of unlimited growth). The peripheral nodal cells each cut off meristematic initials which form laterals of limited growth—a whorl of *branchlets* at each node. Other peripheral nodal cells (e.g., Chara) cut off meristematic initials which form corticating filaments lying along the outside of the internodal cell. This layer is the *cortication*. From other peripheral nodal cells may arise elongated cells, the *stipulodes*, which spread laterally subtending the branchlets (Pl. I, Fig. 3). The branchlets may divide (furcate) into two or more segments or *rays* (Pl. I, Fig. 1-D) at each node to the fourth or fifth order (Nitella), or the branchlets may remain strictly undivided (Chara), and consist of alternating nodes and internodes (except for terminal ecorticated cells). Undivided branchlets produce only one central nodal cell at each node with accompanying peripheral nodal cells. Spine-like processes from these peripheral branchlet nodal cells form *bracts* (Pl. IV, Fig. 4-C) at each branchlet node. The branchlets may be completely or partly corticated or totally ecorticate.

The oogonium or egg-producing cell is fundamentally an apical cell terminal borne on a modified lateral and ensheathed in an encasement of five spirally twisted laterals of limited growth, the *enveloping cells*. At first these are straight, but become tightly spiralled about the oogonium in maturity. The writer employs the term *convolutions* to indicate the resulting spirals apparent on the female gametangium. Each enveloping cell is terminated by one small cell in the tribe Chareae and by two small cells in the tribe Nitelleae, which results in a *coronula* of five cells or ten cells in two superimposed tiers, respectively. The entire female gametangium is known taxonomically as the *oogonium*.

The oogonia generally arise as laterals of the antheridial stalk (see below) in Chara, or from a peripheral nodal cell of a branchlet in Nitella and species of Chara where gametangia are not conjoined. In the genus Chara, elongated cells, the *bracteoles* which often closely resemble bracts, also develop from the antheridial stalk. The *oospore* develops within the enveloping cells of the oogonium. The impression of the enveloping cells on the oospore results in spiralled ridges, the *striae* or *striations*. The outer membrane of the oospores frequently develops characteristic *oospore membrane patterns*.

The biflagellate, spiral *sperms* are produced singly in cells which occur in filaments (*capitular filaments*) borne terminally on laterals (*manubria*) which project inwardly from the centers of four or eight *shield plates*. The shield plates coalesce to form a spheroid structure, and radiating lines (partial septa [modified cells?]) project from the periphery toward the center of each shield plate. The entire spherical male gametangium is known taxonomically as the *antheridium* (Pl. I, Fig. 1-D). The antheridia arise from peripheral nodal cells of branchlets (Chara) or from the apical cell of a ray (Nitella).

The vegetative plants are haploid; meiosis, as far as is definitely known, occurs in the germination of the zygote. Both monoecious and dioecious species occur.

For a more detailed discussion of the morphology, the reader is referred to Fritsch (1935: 447-465).

FAMILY CHARACEAE

The only existing family with characteristics of the class. The family has been divided into tribes and genera based upon both sexual and vegetative characters. A synopsis to this classification is given below.

Coronula of the oogonium consisting of ten cells in two tiers—Tribe NITELLEAE
Ganterer (1847: 8), *pro parte, emend.* Leonhardi (1863: 69).

Antheridia apical on a ray in the furcation of the branchlets—Genus NITELLA
C. A. Agardh (1824: xxvii), *pro parte, emend.* Braun (1849a, b: 195, 292).

Antheridia lateral at the furcation of the branchlets—Genus TOLYPELLA
(Braun, 1849a: 199) Leonhardi (1863: 72).

Coronula of the oogonium consisting of five cells in one tier—Tribe CHAREAE
Leonhardi (1863: 72).

Stipulodes at the base of the branchlets lacking.

Bracts present, one to two at a node—Genus NITELLOPSIS Hy (1889: 397).

Bracts absent—Genus PROTOCHARA Womersley and Ophel (1947: 311).

Stipulodes at the base of the branchlets present, although sometimes rudimentary.

Oogonia normally situated below antheridia; axes ecorticate—Genus LAMPROTHAMNIUM Groves (1916: 336), *emend.* Ophel (1947: 322).

Oogonia normally lateral with respect to antheridia, an oogonium situated between two antheridia; axes corticate—Genus Lychnothamnus (Ruprecht, 1845: 11) *pro parte*, Leonhardi (1863: 72).

Oogonia situated above the antheridia; axes corticate or ecorticate—Genus CHARA Vaillant *ex* Linnaeus (1754: 491).

Womersley and Ophel (1947) recently described a new genus *Protochara*. This genus is founded upon *P. australis* Wom. & Oph., a new species described in the same article. *Nitellopsis inflata* Filarszky & Allen was transferred to the new genus in the combination *P. inflata* (Fil. & Allen) Wom. & Oph. Ophel (1947), in another article, emended *Lamprothamnium* Groves, and included *Chara macropogon* Braun in the combination *Lamprothamnium macropogon* (Braun) Ophel. He also suggested removing *L. Hansenii* Sonder to *Chara Hansenii* (Sonder) Ophel. The material and evidence to support these changes have not as yet been examined by the present writer.

Of the six or seven known genera, three occur in North America; namely, *Chara*, *Nitella*, and *Tolypella*. Of these, *Chara* and *Nitella* are represented in our region; although *Tolypella* is known to occur as near as the Finger Lakes of New York, and has been recorded from Vermont.

Genus Nitella

Branches commonly two or more at a node. Branchlets one or more times furcate into two or more rays (Pl. I, Fig. 1-D) at each furcation. The ultimate rays of a branchlet beyond the last furcation are known as *dactyls* (Pl. I, Fig. 1-D). In many species a small terminal cell occurs on the dactyl, and is known as a *muco* (Pl. III, Fig. 2-A). In some species the fertile branchlets are greatly reduced and

PLATE II



modified; in others the fertile branchlet whorls and the branch upon which they are borne are reduced and form more or less dense *heads* (Pl. II, Fig. 2). Fertile heads may be enveloped in *mucus*—a hyaline, gelatinous material. An oogonium arises from peripheral nodal cells of a branchlet, thus replacing a ray, and projects laterally below the whorl of rays. An antheridium occurs terminally on a ray amid the whorl of rays of the next order, replacing the apical cell of the ray. Cells of the coronula of the oogonium ten, in two superimposed tiers of five cells each. Oospores are laterally compressed.

In our region, specimens of *Nitella* can be immediately distinguished from *Chara* in the field by the obvious character that the branchlets are divided, whereas they are strictly undivided in *Chara*.

1. *Nitella flexilis* (Linn., *pro parte*) C. A. Agardh, Syst. Alg., p. 124. 1824.

Chara flexilis Linn., Spec. Plant., p. 1157. 1753.

References for the region: *N. flexilis*: Owen (1888: 74); Croasdale (1935: 94); var. *subcapitata*: Owen *l.c.*

(Plate II, Fig. 1; Plate III, Fig. 1)

Plant monoecious, 20 cm. high, diffuse but robust. *Branchlets* about eight in a whorl, once furcate into two long dactyls (one occasionally lacking). *Dactyls* (1-) 2², equal to or exceeding the primary ray in length, terminated by a sharp-pointed mucronate tip (not a distinct cell). *Gametangia* generally aggregate, (1-) 2 (-3) oogonia and an antheridium at each fertile node. *Oogonia* 850-875 μ long by 690-710 μ broad; coronula about 75 μ broad by 36 μ high, ultimately deciduous; convolutions 7. *Oospores* 530-550 μ long by 472-500 μ broad, black; striae apparently 5; membrane smooth. *Antheridia* 340-415 μ (immature; 515-555 μ in *R. D. Wood 2014*) in diameter. (Descr. from *R. D. Wood 2013* (RDW).)

Variations among the local forms result from differences in development of vegetative structures. In size, plants vary up to 35 cm. high. The dactyls vary from short ($\frac{1}{6}$ length of primary ray) in *Maria Owen 3*, to greatly elongated (longer than primary ray) in *R. D. Wood 2014*. The rays vary from those which are all equal in length and give a distinctly regular appearance to the whorls as in *R. D. Wood 2030*, to those which are very unequal in length and give a ragged appearance to the whorls as in *T. Morong 3*. The branchlets vary from elongate in *R. D. Wood 2014* giving loose whorls, to very short in *T. Morong 2b* giving ap-

² G. O. Allen (corresp., 1948) reports that three dactyls occasionally occur in British specimens.

PLATE II

Drawn from the indicated herbarium specimens which are extant in the writer's herbarium. $\times 0.5$.

FIGURE 1. *Nitella flexilis* (L., *pro parte*) C. Agardh. (2030.)

FIGURE 2. *Nitella Morongii* Allen *emend.* Wood. (*E. T. Moul 3173*.)

FIGURE 3. *Nitella megacarpa* Allen. (2060.)

FIGURE 4. *Nitella transilis* Allen. (2021.)

FIGURE 5. *Chara delicatula* C. Agardh. (2058.)

FIGURE 6. *Chara canescens* Lois. (2027.)

FIGURE 7. *Chara Braunii* Gmelin. (2004.)

pearance of reduced clumps isolated on elongated internodes. The fertile whorls are generally similar to the sterile whorls, but in *W. R. Taylor* (17085 WRT) the heavily fertile whorls are somewhat reduced into loose heads. The color of dried specimens is generally translucent greenish brown, but certain specimens become opaque dark brown as in *T. Morong* 1 and thus suggest *N. opaca* (but our specimens are monoecious!). These extreme characteristics appear to occur in random combination, and could permit designation of easily ten or more different forms; but none of the combinations seem sufficiently constant to merit assignment to varietal status. Allen (1871: 9; 1880: 11) and Owen (1888: 74) have reported those forms in which fertile whorls are somewhat reduced as var. *subcapitata* (Hartm.) Groves (= var. *nidifica* Groves, H. & J.), and Allen (1880: pl. 5) assigned forms with extremely shortened branchlets and stout internodes to var. *crassa* Braun and to form *superne brachyphylla* (in herb. NY).

N. flexilis is easily distinguished in the field, as it is the sole species in our local flora in which the branchlets are only once divided. However, occasional sterile branchlets may not be furcate, and inspection of the fertile whorls may be necessary to demonstrate this feature.

Illustrations: Allen (1892: pl. 6, 6a, 6b); Groves & Bullock-Webster (1920: pl. 8); Migula (1897: 133; 1925: 214, fig. 4, 5); Woods (1894: pl. 26).

Exsiccatae: Phyc. Boreali-Amer., No. 1435, 1691; Char. Amer. Exsicc., No. 28, 29, 30.

Localities: CAPE COD: Weeks Pond—*R. D. Wood* 2016, July 26, 1947 (RDW); CUTTYHUNK: Clubhouse Pond—*R. D. Wood* 2030, July 31, 1947 (RDW, NY); Sheep Pond—*W. R. Taylor*, June 28, 1932 (17085 WRT, NY); Sheep Spring—*W. R. Taylor*, June 27, 1933 (16981, 17010 WRT); July 3, 1934 (16769 WRT); MARTHA'S VINEYARD: Chilmark Pond—*R. D. Wood* 2014, July 20, 1947 (RDW, NY); Tiasquam Dam—*R. D. Wood* 2013, July 20, 1947 (RDW); NANTUCKET: Cato's Pond—*T. Morong* 3, July 22, 1887 (NY); New Lane Pond, pond west of New Lane and S. of Grove Lane—*Grace W'yatt*, Sept. 4, 1947 (RDW); Polpis—*Mary Owen* 3, July, 1879 (NY), as var. *subcapitata*; R. R. Track Pond, pool E. of town [Nantucket] near R. R. tracks—*T. Morong* 1, July 16, 1887 (NY); Sesachacha, drainage ditch S.W. of pond—*Grace W'yatt*, Aug. 12, 1947 (RDW); Weweeeder Pond—*T. Morong* 2b, July 16, 1887 (NY); July 15, 1887 (NY); NONAMESSET: South Pond—*Hannah Croasdale* 55, June 15, 1935 (MBL, preserved; 2012 RDW); PASQUE: West End Pond—*W. R. Taylor*, July 2, 1941 (20840 WRT, FH); ? Pond, west end of island—*W. R. Taylor*, July 3, 1940 (19691 WRT).

2. *Nitella megacarpa* Allen, Characeae Americanae Exsiccatae, No. 3. 1880.

Nitella microcarpa subsp. *megacarpa* (Allen) Nordstedt *apud* Braun, Fragmente einer Monographie der Characeen, p. 73. 1882.

References for the region: *N. mucronata*: Owen (1888: 74 [pl. not seen]); var. *gracillima* Groves & B.-W.: Croasdale (1935: 94).

(Plate II, Fig. 3; Plate III, Fig. 4)

Plant monoecious, 25 cm. high, robust, bright green. *Branchlets* (4-) 5-6 (-7) in a whorl, up to 3.5 cm. long, spreading widely; whorls at apex of main axes partly convergent and forming a rather broad, terminal clump; branchlets bear gametangia throughout the season, 3-4 times furcate into 5 secondary rays, 2-3 tertiary rays, 2-3 quaternary rays, and occasionally 2-4 quinary rays. *Dactyls* 2-3, 2-3 (occasionally 4-more) -celled; ultimate cell a conical mucro 37-74 μ long; basal cell 100-500 μ long; intermediate cells (when present) generally one or two subcylindri-

cal cells a little longer than broad. *Gametangia* borne on branchlets of all whorls, present throughout the season, solitary to aggregated, not enveloped in mucus. *Oogonia* about $710\ \mu$ long by $510\ \mu$ broad, 1–2 (–3 or more) at a branchlet node; coronula about $25\ \mu$ long by $36\ \mu$ broad; convolutions 9. *Oospores* about $420\ \mu$ long by $370\ \mu$ broad, yellow when immature, darkening to deep brown on maturity, often nearly spherical; striae 5–6; membrane strongly reticulate; reticulae 2.2 – $3.6\ \mu$ in diameter, subquadratic, 9–11 across a fossa. *Antheridia* 265 – $445\ \mu$ in diameter. (Descr. from *R. D. Wood 2060* (RDW).)

This large, bright *Nitella* is by far the most beautiful species in the region, and one which cannot be confused once it has been seen in the field. In size, the plants vary up to nearly 40 cm. high.

Nordstedt (1882) and Zaneveld (1940) included *N. megacarpa* within the limits of *N. microcarpa* Braun. Our form certainly is very similar in most fundamental respects to this species, and is doubtlessly very close phylogenetically. The local form differs in being a much larger plant with much larger gametangia. The greater general size suggests polyploidy from *N. microcarpa*. If this proves to be the case, the erection of a distinct species is almost required. Furthermore, the writer has seen no specimens which exhibit complete serial intergradation between the two forms. Therefore, until intergrades are seen, or until cytological investigations are completed, the writer prefers to consider *N. megacarpa* Allen a distinct species in agreement with G. O. Allen (corresp., 1948).

A characteristic feature of this plant is the manner in which the main axis is easily separated at the nodes. As a result, specimens are commonly collected, especially with plant hooks, which consist of only the terminal clump of whorls of branchlets. These fragments do not give the impression of massiveness characteristic of the plant.

Illustrations: No habit sketches of this species have been published previously.

Exsiccatae: Phyc. Boreali-Amer., Fasc. 32, No. 1588, Fasc. E, No. CII; Char. Amer. Exsicc., No. 3.

Localities: CAPE COD: Ashumet Pond—*I. F. Lewis*, Sept. 4, 1926 (WRT); Leech Pond—*C. W. Palmer*, July 27, 1936 (17199 WRT); Summerfield Pond, South—*C. C. Jao*, Sept. 4, 1933 (16771 WRT); Weeks Pond—*Hannah Croasdale*, July 28, 1947 (2019 RDW, MBL, MSD, ETM); *R. D. Wood 2031*, Aug. 1, 1947 (RDW), 2060, Sept. 6, 1947 (RDW); Wood Pond—*W. R. Taylor*, July 2, 1921 (3668 WRT).

3. *Nitella Morongii* Allen, Bull. Torrey Bot. Club 14: 214. 1887; *emend.*

Wood, *Rhodora* 51 (602): 16. 1949.

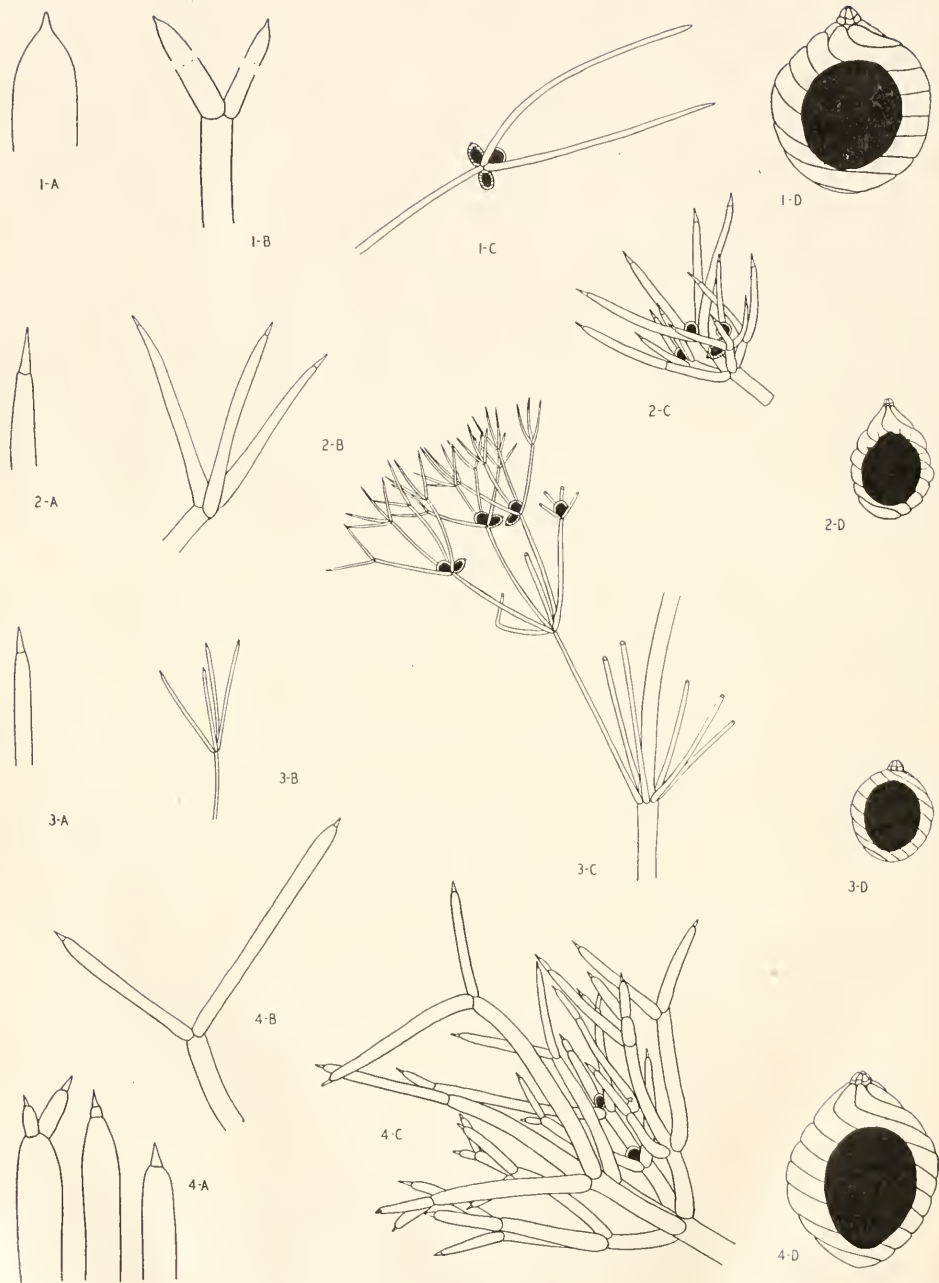
Nitella maxceana Allen, *spec. dub.*, Char. Amer. 2(3): 27. 1896.

References for the region: *N. batrachosperma*: Owen (1888: 74); *N. gracilis*: Halsted (1879: 176), Croasdale (1935: 95); f. *brachyphylla*: Collins in Phyc. Boreali-Amer., No. 1195; *N. maxceana*: Allen, l.c.; *N. Morongii*: Owen, l.c., Allen, l.c., Wood, l.c.

(Plate II, Fig. 2; Plate III, Fig. 2)

Plant monoecious, 14 cm. high, delicate, and with characteristic heads. *Branchlets* of two types, including: (1) the normally expanded sterile or lightly fertile branchlets, and (2) the greatly reduced fertile branchlets. *Sterile branchlets* 2–5 in a whorl, 15–26 mm. long, 1–2 times furcate into 3–5 secondary rays, 2–3 tertiary rays. *Dactyls of sterile branchlets* 2–3, 2-celled, the ultimate cell a conical mucro

PLATE III



which is early deciduous. *Fertile branchlets* 6–7 in a whorl, twice furcate, greatly reduced, 1–3 mm. long; 3–5 such reduced whorls borne on a reduced branch, the entire complex resembling a dense head; heads apparently axillary in sterile whorls or terminal, enveloped in mucus. *Dactyls of fertile branchlets* 2 (–3), 2-celled, one commonly shorter than the other, terminated by an elongated mucro. *Gametangia* solitary, an oogonium and an antheridium at all fertile branchlet nodes, enveloped in a weak mucus.³ *Oogonia* 290–386 μ long by 210–288 μ broad; coronula $35 \times 35 \mu$. *Oospores* 238–268 μ long by 180–210 μ broad; striae of 5 prominent ridges; membrane roughened with anastomosing lines, almost appearing very finely reticulate. *Antheridia* somewhat flattened longitudinally, 134–148 μ long by 174–179 μ broad, short stipitate. (Descr. from *E. Moul 3173* (RDW).)

Specimens which have been collected since 1888 are quite consistently of a diffuse form as in *E. Moul 3173*, described above. A compact form as in the TYPE COLLECTION *T. Morong*, July 21, 1887, has very short branchlet rays, more fertile whorls at a fertile branch, and early deciduous sterile branchlets. This modification is thought to be the result of extreme ecological conditions exerted by constant trampling by the feet of animals and the muddy water (cf. Wood, 1949). In size, some plants reach 20 cm. high.

Illustrations: Allen (1894: pl. 16), excellent for the original material, but not as emended. Exsiccatae: Phyc. Boreali-Amer., No. 1195 as *N. gracilis*, 1382.

Localities: CAPE COD: Golf Pond—*G. M. Gray*, July, 1931 (17006 WRT); *W. R. Taylor*, July 6, 1917 (2293 WRT); *Hannah Croasdale*, June 24, 1935 (17078 WRT, NY, 950899 F, RDW); Harper Pond—*E. T. Moul 3173*, July 7, 1947 (ETM, RDW, NY); *Urda K. Wood*, July 11, 1947 (2011 RDW, NY); Nobska Pond—*W. A. Setchell* and *W. J. V. Osterhout 644*, July 15, 1894 (MBL, FH, 315652 UC); *W. A. Setchell*, July 15, 1893 (P. B.-A. No. 1195, as *N. gracilis*, NY); ? Pond, Woods Hole—[*Coll.?*], 1883 (NY); NANTUCKET: Maxey's Pond—*T. Morong*, July 7, 1887 (NY, Type of *N. maxicana* Allen); Siasconset, small pond south side of old Sconset Road, opposite "Bloomingdale"—*L. L. Dame*, July, 1886 (NY, in one P. B.-A. packet No. 1382); in a very muddy pool on the roadside near Siasconset—*T. Morong 4*, July 21, 1887 (FH, NY, TYPE COLLECTION); in a small pool near Siasconset—*F. S. Collins*, Aug. 23, 1896 (P. B.-A. No. 1382, NY, BRU); NAUSHON: Petchett (or Peckett [?]) Pond—*Hannah Croasdale*, July 5, 1946 (MBL, preserved).

4. *Nitella transilis* Allen, Char. Amer. 2(3): 24. 1896.

References for the region: none known.

³ Allen (1888) in his synopsis enters *N. Morongii* Allen under "heads not enveloped in mucus," but this point is reported with some question later (Allen, 1894: 15).

PLATE III

All figures have been drawn with the aid of a camera lucida from specimens preserved in 2–3 per cent formalin. The indicated specimens are extant in the writer's herbarium.

FIGURE 1. *Nitella flexilis* (L. *pro parte*) C. Agardh. 1-A, tip of terminal branchlet cell, $\times 35$. 1-B, dactyls, $\times 14$. 1-C, fertile branchlet node, one antheridium and two oogonia, $\times 14$. 1-D, oogonium, $\times 35$. (2013.)

FIGURE 2. *Nitella Morongii* Allen *emend.* Wood. 2-A, tip of dactyl with terminal mucro, $\times 35$. 2-B, dactyls, $\times 14$. 2-C, fertile branchlet, $\times 14$. 2-D, oogonium, $\times 35$. (*Urda K. Wood 2009*.)

FIGURE 3. *Nitella transilis* Allen. 3-A, tip of dactyl with terminal mucro, $\times 35$. 3-B, dactyls, $\times 14$. 3-C, axial node and part of one branchlet, $\times 14$. 3-D, oogonium, $\times 35$. (A and B from 2015; C and D from 2059.)

FIGURE 4. *Nitella megacarpa* Allen. 4-A, tips of dactyls showing variations in form of mucros, $\times 35$. 4-B, dactyls, $\times 14$. 4-C, fertile branchlet (a very small specimen), $\times 14$. 4-D, oogonium, $\times 35$. (2019.)

(Plate II, Fig. 4; Plate III, Fig. 3)

Plant monoecious, 12 cm. high, very fine and delicate. *Branchlets* 6-7 in a whorl (branches occasionally two at a node), 3-4 times furcate into 6 secondary rays, 5 tertiary rays, and 4 (-5) quaternary rays; quinary rays when present 2-3; branchlets usually exceeding the axial internodes in length, primary rays about $\frac{3}{5}$ the total length of the branchlet. *Dactyls* 2-3 (-5), 2-celled. *Gametangia* solitary, an antheridium and an oogonium borne on the second and third, rarely at the first, branchlet nodes, enveloped in mucus. *Oogonia* about $309\ \mu$ long by $240\ \mu$ broad; convolutions 7-8. *Oospores* about $276\ \mu$ long by $170\ \mu$ broad; striae of 6 ridges; membrane strongly reticulate, reticulae 10-12 across the fossae. *Antheridia* about $178\ \mu$ in diameter, stipitate on stalks about $37-59\ \mu$ long; markings on shield extending about half way to base of manubrium, irregular. (Descr. from *R. D. Wood 2015* (RDW).)

The local form of this species is very constant, and apparently does not exhibit modifications found in other parts of eastern North America. In size it reaches 16 cm. in height. Some specimens might be confused with *N. Morongii* because of the size and occurrence of mucus.

Whether *N. transilis* Allen is sufficiently distinct to be separated from *N. tenuissima* (Desv.) Kütz. has been discussed (corresp., 1946-1948) with G. O. Allen. The European *N. tenuissima* f. *gracilior* (L. Chevallier, Aug. Sept. 1893. *Gallia occidentalis: In stagnis circa "Bazouges"* (7185 WRT)) approaches *N. transilis* rather closely. Other intergrades of European *N. tenuissima* exhibit diffuse branching resembling *N. transilis*. Further significant information is accumulating in the writer's herbarium in the form of a series of intergrades from various stations in New England. It seems probable that our specimens may well be included under *N. tenuissima*. At present, the characters which seem sufficient to retain it as a separate species from *N. tenuissima* include: (1) the very regular diffuse whorls, (2) the branchlets exceed the internodes in length, (3) the rather frequent occurrence of gametangia at the first branchlet node, and (4) the stipitate antheridia.

Illustrations: Allen (1896: pl. 23).

Exsiccatae: Char. Amer. Exsicc., No. 31, sub. nom. *N. tenuissima* f. *longifolia elongata*.

Localities: CAPE COD: Ashumet Pond—*M. S. Doty* & *L. Spiegel* 7470, Aug. 17, 1948 (MSD, RDW); John Pond, Mashpee—*G. W. Prescott* 5, Aug. 30, 1937 (17973 WRT); Weeks Pond—*Hannah Croasdate*, July 14, 1946 (6615 MSD), July 28, 1947 (2021 RDW, ETM, MBL, MSD, NY); *R. D. Wood* 2015, July 26, 1947 (RDW, NY); *R. D. Wood* 2059, Sept. 6, 1947 (RDW, mature).

Genus *Chara*

Branches formed occasionally at nodes. Branchlets not furcate, divided by nodes and internodes into a continuous series of *articulations*; the terminal two or more articulations may lack nodes, and be merely separated by cell walls. Spine-like processes (*bracts*, Pl. IV, Fig. 4-C) arise from peripheral cells of the branchlet nodes, and form a more or less distinct whorl at sterile and fertile nodes. Branchlet articulations corticated, partly corticated, or uncorticated. Main axes corticated or uncorticated (*ecorticate*, Pl. I, Fig. 3-A, 4-A). Cortication varies from *triplostichous* (Pl. I, Fig. 3-D, 4-D) in which the secondary cortical cells

are nearly equal in length to the primary cortical cells, thus forming two series of cells between each primary cortical series (and three times as many cortical filaments as branchlets at an adjacent node); to *diplostichous* (Pl. I, Fig. 3-C, 4-C) in which the secondary cortical cells are about half the length of the primary cortical cells so that the secondary cells from adjacent primary cortical cells series lie end to end forming one continuous series of cells between each primary cortical series (and thus twice as many cortical filaments as branchlets at an adjacent node); to *haplostichous* (Pl. I, Fig. 3-B, 4-B) in which the secondary cortical cells do not become enlarged, thus only the primary cortical cells are apparent in the cortication (and thus the same number of cortical filaments as branchlets at an adjacent node). The details of cortication are best seen by inspecting the cortication of very young axial internodes (*vide* G. O. Allen, corresp., 1948) before they become unduly elongated. One series of stipulodes (*haplostephanous*, Pl. I, Fig. 3-A) or two series (*diplostephanous*, Pl. I, Fig. 3-C) may develop from axial nodal peripheral cells and subtend the branchlets. Cortical nodal cells divide longitudinally into an inner and outer cell. The outer cell may merely swell into a *papillus* or *spine*, or may cut off several elongated processes (*spines*) in groups of 2-5 (*fascicled*, Pl. IV, Fig. 2-B). Gametangia borne primarily at the branchlet nodes; in monoecious species the antheridium is generally directly below the oogonium (*conjoined*), or isolated at a separate node (*sejoined*); in dioecious species borne on separate plants. Antheridia arise from peripheral nodal cells of branchlet nodes. Oogonia (in conjoined monoecious species) arise from laterals of antheridial stalk, on the abaxial side; spine-like processes (*bracteoles*, Pl. I, Fig. 2-C) which develop from cells of the antheridial lateral appear to subtend the oogonium. These may closely resemble the bracts. Starch bulbils regularly formed on rhizoids of certain species. Oospores terete, not laterally compressed.

5. *Chara Braunii* Gmelin, Fl. Badensis Alsatica 4: 646. 1826.

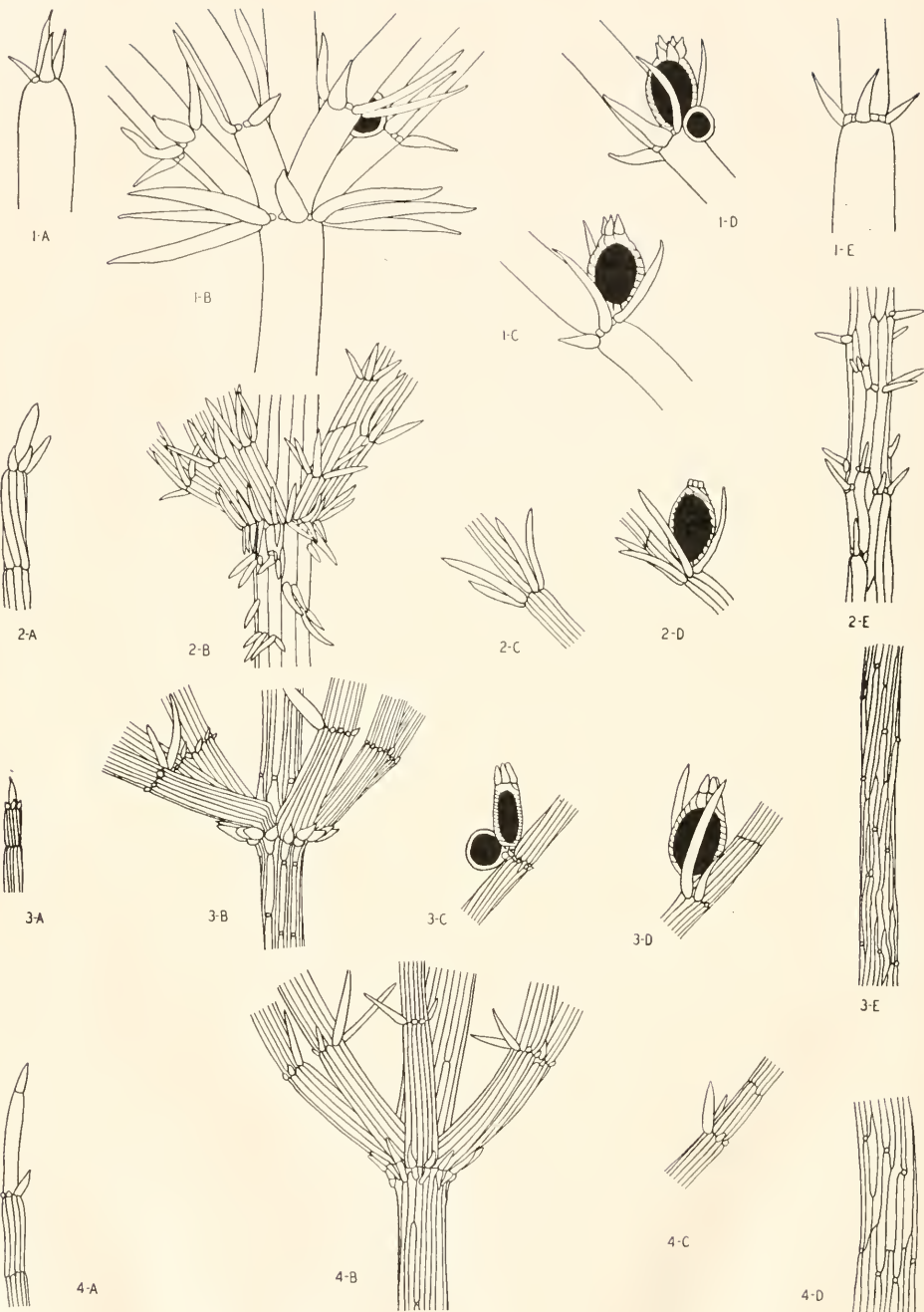
Chara coronata Ziz. (ined., c. annum 1814); Braun, Ann. Sci. Nat., ser. II, 1: 353. 1834.

References for the region: *C. coronata*: Owen (1888: 75); var. *Schweinitzii*: Halsted (1879: 181); Owen, *l.c.*

(Plate II, Fig. 7; Plate IV, Fig. 1)

Plant monoecious, totally ecorticate, 20 cm. high. *Branchlets* 8-9 in a whorl, widely spreading to closely convergent; articulations of branchlets (3-) 4 (-5), first and second nodes generally fertile; bracts 300-455 μ long to greatly reduced; terminal internodal cell occasionally somewhat inflated, terminated by a crown of 3 mucro-like cells, each 150-274 μ long. *Stipulodes* in one series (*haplostephanous*), 730-1035 μ long, alternating with the branchlets, spreading. *Gametangia* at first and second branchlet nodes, fundamentally conjoined, but antheridia frequently early deciduous; occasionally the sex organs geminate (2 oogonia and antheridia at a node). *Oogonia* 660-960 μ long by 384-490 μ broad; coronula about 150 μ long by 90 μ broad, the cells with short, divergent tips; convolutions 10; bracts about equal in length to the oogonia. *Oospores* 470-590 μ long by 259-340 μ broad, black; striae 8-10; membrane smooth and nearly opaque. *Antheridia* 268-282 μ in diameter. (Descr. from Hannah Croasdale (2008 RDW); details of the gametangia from Urda K. Wood, July 16, 1947.)

PLATE IV



Variations among the local forms result from differences in development of vegetative structures. In size, some plants reach 35 cm. in height. The general plant structure varies from diffuse, attenuate forms, as in *Hannah Croasdale* (2020 RDW), typical of relatively deep, quiet water to short, compact forms as in *R. D. Wood* 2033, characteristic of shallow littoral habitats. The bracteoles vary from shorter than mature oogonia in *R. D. Wood* 2005 to $1\frac{1}{2}$ to 2 times as long in *Hannah Croasdale* (2020 RDW). The crown of end cells terminal on branchlets varies in length from equal to the diameter of the branchlet in *R. D. Wood* 2005 to less than half the diameter in *R. D. Wood* 2029. The relative lengths of the branchlet articulations vary from those in which the basal internode is about half the length of the others in *R. D. Wood* 2005, to those in which this articulation is extremely short in (*Coll.*?, Aug.), to those in which the terminal articulation is shortened and sometimes swollen in *B. F. D. Runk* 457. The elongation of the bracts at the primary branchlet nodes varies from about equal to other bracts in *R. D. Wood* 2033 to those in which these bracts are twice the length of the bracts of other nodes in *B. F. D. Runk* 457. The stipulodes vary greatly in length on the same specimen, but are characteristically short in *Hannah Croasdale* (2008 RDW) and long in *B. F. D. Runk* 457. The apparent general trend is for plants growing in shallow littoral zones to be tufted, form tightly connivent whorls, have shortened branchlet articulations and elongated bracts and stipulodes. Plants of deep, quiet water tend to be diffuse, have elongated branchlet articulations and reduced bracts, bracteoles, and stipulodes. These appear to be ecological variants, and range from what has been considered var. *Schweinitzii* Braun, in which bracteoles exceed mature oogonia in length, to var. *Braunii* (Braun) Zanev., in which bracteoles are equal to or shorter than mature oogonia. Oospores vary in length from 450–600 μ and thus are intermediate between the limits of var. *Schweinitzii* (550–650 μ) and var. *Braunii* (420–550 μ) as delimited by Zaneveld (1940: 139). It is thus concluded again (cf. Wood, 1947: 250; Allen, 1882b) that attempts to differentiate varieties of this species collected from various localities of North America are impossible in view of the facts. The writer has made a practice of annotating extreme specimens as approaching either the one variety or the other, but emphasizes that this is but a convenient manner of indicating differences in expression of the

PLATE IV

All figures have been drawn with the aid of a camera lucida from specimens preserved in 2–3 per cent formalin. The indicated specimens are extant in the writer's herbarium. $\times 14$.

FIGURE 1. *Chara Braunii* Gm. 1-A, terminal cells of branchlet. 1-B, axial node showing stipulodes at base of branchlets and lack of cortication. 1-C, oogonium. 1-D, oogonium subtended by antheridium. 1-E, bracts at sterile branchlet node. (*Hannah Croasdale* (2008 RDW).)

FIGURE 2. *Chara canescens* Lois. 2-A, terminal cells of branchlet. 2-B, axial node showing two rows of stipulodes, spines in fascicles, and haplostichous cortication. 2-C, bracts of sterile branchlet node. 2-D, oogonium at fertile branchlet node. 2-E, axial cortication. (2004.)

FIGURE 3. *Chara delicatula* C. Agardh. 3-A, terminal cells of branchlet. 3-B, axial node showing one row of stipulodes and diplostichous tylacanthous cortication. 3-C, fertile branchlet node with oogonium and antheridium. 3-D, fertile branchlet node with an oogonium. 3-E, axial cortication. (2002.)

FIGURE 4. *Chara delicatula* C. Agardh var. *barbata* Groves & B.-W., *prox.* 4-A, terminal cells of branchlet showing elongated penultimate cell. 4-B, axial node showing well-developed two rows of stipulodes, and nearly isodiametric primary and secondary cortical cells. 4-C, bracts at sterile branchlet nodes. 4-D, axial cortication. (2024.)

vegetative characteristics. One peculiar specimen was found (*Coll.*?, Aug.), which had a definite glomerate appearance. This resulted from the development of a somewhat reduced branch at each node, combined with elongation of bracts and stipulodes, and typical compactness of shallow-water forms. No further collections of this form have been seen; and, until other specimens have been reported, it will be considered an anomaly.

This species is the favorite local Charad for physiological investigation, and investigators commonly mistake the plants for a *Nitella*. There should be no confusion once it is recognized that this species is an ecorticate *Chara*, and that it is the only local species of Characeae which is ecorticate, has stipulodes at the base of the branchlets, and in which the branchlets are not divided. The latter two characters easily distinguish the species in the field.

Illustrations: Allen (1882b); Groves & Bullock-Webster (1924: pl. 26); Woods (1894: pl. 30); Migula (1897: 324; 1925: 226, fig. 1, 2).

Exsiccatae: Phyc. Boreali-Amer., No. 822; 1383 as *C. Schweinitzii*; Amer. Alg., No. 256, 529; Char. Amer. Exsicc., No. 8, 12, 13, 14.

Localities: CAPE COD: Chara Haven—*R. D. Wood* 2005, July 7, 1947 (RDW, ETM); Chara Pond—*B. F. D. Runk* 406, June 20, 1941 (109977+ F, RDW); Desmid Haven—*E. T. Moul*, July 11, 1945 (ETM); Harper Pond—*J. J. Copeland*, July, 1928 (13800 WRT); Ice House Pond—*M. S. Doty*, July 10, 1946 (6624 MSD); Little Pond—*R. D. Wood* 2033, Aug. 1, 1947 (RDW, NY); Nobska Pond—*W. G. Farlow*, Sept. 15, 1876 (FH); Oyster Pond—*Urda K. Wood*, July 16, 1947 (RDW, preserved); Salt Pond—*R. D. Wood* 2034, July 30, 1947 (RDW); "Sandwich Pond," pond on W. side of road, route No. 6, E. of Sandwich—*R. D. Wood* & *Urda K. Wood* (2007 RDW); Weeks Pond—*Hannah Croasdale*, July 14, 1946 (6613 MSD), July 28, 1947 (2020 RDW); *R. D. Wood* 2017, July 26, 1947 (RDW); CUTTYHUNK: Gosnold Pond—*W. R. Taylor*, July 6, 1921 (3670 WRT); Sheep Pond—*I. F. Lewis* (MBL); *W. R. Taylor*, July 9, 1925 (10173 WRT, MICH); *R. D. Wood* 2027 (RDW) and 2029 (RDW, NY), July 31, 1947; NANTUCKET: Long Pond—*W. H. Sheldon*, Aug. 7, 1934 (MMA); Sesachacha Pond—[*coll.*?], Aug. (NY); Weweeder Pond—[*T. Morong*] 2a, July 15, 1887 (NY); NAUSHON: French Watering Place—*W. R. Taylor*, July 12, 1921 (3669 WRT); July 13, 1922 (7585 WRT); PASQUE: West End Pond—*B. F. D. Runk* 457, July 1, 1941 (1099770 F, RDW); ? Pond, small pool—2nd or 3d pool south of West End Pond—*W. R. Taylor*, July 2, 1941 (FH, 20841 WRT); ? Pond, smaller pond near the southwest end of island—*Hannah Croasdale*, July 6, 1933 (16982 WRT); PENIKES: Typha Pond—*Hannah Croasdale*, July 6, 1947 (2008 RDW, MBL, MSD).

6. *Chara canescens* Desvaux et Lois. *apud* Loiseleur-Deslongchamps,⁴ Not. Fl. France, p. 139. 1910.

Chara crinita Wallr., Annus Bot., p. 190. 1815.

Chara crinita var. *americana* Allen, Char. Amer., Part 2, plate 2. 1879.

References for the region: *Chara crinita*: Owen (1888: 75); *i. leptosperma*: Allen (1880: 5; 1882a: 41); Owen, l.c.; *C. canescens*: Croasdale (1935: 95).

(Plate II, Fig. 6; Plate IV, Fig. 2)

Plant dioecious, 12 cm. high, densely hirsute. *Main axes* haplostichous, cortical nodes bearing spines solitary to fascicles of 2–5, one secondary cortical cell commonly apparent at cortical nodes and extending $\frac{1}{8}$ to $\frac{1}{4}$ the way to the next node (some-

⁴ Loiseleur-Deslongchamps has consistently been given the credit for this species. In a footnote (p. 135), he stated that Desvaux had recognized several new species in the genus *Chara* and desired that they be published for him pending a more extensive comparative examination of the species. The present species, however, was entitled "*Chara canescens*. N."; and since "N." indicates *nobis* (by us), both men are necessarily recognized in the author citation.

times greatly developed so as to give a nearly diplostichous appearance). *Branchlets* 6–8 in a whorl, articulations 4–6, corticated except for terminal 1–2 cells, terminal cell rounded at tip; bracts varying widely in length, blunt. *Stipulodes*, diplostephanous (in 2 series), 2 pairs at each branchlet, upper series 297–447 μ long, generally exceeding the lower series in length, blunt. *Gametangia* borne on separate plants, i.e., dioecious. *Oogonia* about 890 μ long by 442 μ broad, borne regularly at first branchlet node, occasionally at second and third; convolutions 10; coronula short and broad, truncate, 86 μ tall by 147 μ broad; bracteoles blunt to acute, about equal to oogonia in length. *Oospores* about 590 μ long by 318 μ broad, black; striae about 10; membrane opaque. *Antheridia*, none seen on local specimens. (Descr. from *R. D. Wood 2004* (RDW).)

Little variation is exhibited among the local specimens collected. In size, some plants reach 20 cm. in height. The spines on the axes vary from very abundant and nearly twice as long as the diameter of the axis in *T. F. Allen*, Aug. 30, 1895, to rather sparse and shorter than the diameter of the axis in *R. D. Wood 2004*. Bracteoles vary from about equal in length to the mature oogonia in *R. D. Wood 2004*, to one to two times the length in *F. S. Collins*, July 25, 1882. The differences in expression of length of stipulodes, bracts, and spines appear to be more directly correlated with local physiochemical factors, particularly salinity, rather than depth as in *C. Braunii*. This follows logically since most local specimens of *C. canescens* have been found growing in water less than one foot deep. All the local specimens seen exhibited the angular oospores which characterize the form *leptosperma* Braun (1834: 356) as described by Allen (1882a: 41, pl. XVIII), but the present writer prefers to temporarily ignore forms as nomenclatural entities.

This species is highly characteristic in its very spinous appearance of the main axes. It is restricted to brackish-water ponds. Salinity determinations by means of silver nitrate titrations on fresh samples of water from various habitats during July and August, 1947, showed extremes of 4 to 22 ‰ NaCl, with the optimum vegetative development at about 10 ‰ NaCl.

Although no antheridia were discovered on local specimens, a very similar monoecious species, apparently *C. evoluta* Allen, was found in 1948 by Dr. V. I. Cheadle and Dr. E. A. Palmatier in Little Compton, Rhode Island, just across the state line of Massachusetts.

Illustrations: Allen (1879: pl. 2; 1880: pl. 2); Mägula (1897: 352, 353; 1925: 226, fig. 6); Groves & Bullock-Webster (1924: pl. 27).

Exsiccatae: Phyc. Boreali-Amer., No. 823 as *C. crinita*.

Localities: CAPE COD: Chara Pond—*J. M. Fogg, Jr.*, June 29, 1925 (MBL, 10240 WRT); *W. R. Taylor*, July 30, 1937 (17937 WRT, NY, FH); *H. T. Croasdale*, July 14, 1946 (6612 MSD); *R. D. Wood 2004*, June 28, 1947 (RDW, MBL, MSD, ETM); Little Pond—*F. S. Collins*, July 25, 1882 (19776 WRT, RDW, NY, 132461 UC; dupl. herb. *T. F. Allen*, as *C. crinita*); *W. A. Setchell* and *W. J. V. Osterhout*, Aug. 17, 1895 (MBL); *F. S. Collins*, July, 1886 (Y); July 25, 1882 (NY, 132461 UC, RDW); Salt Pond—*Urda K. Wood*, June 28, 1947 (2003 RDW); *R. D. Wood 2023*, July 30, 1947 (RDW, NY); ? Pond, Woods Hole—*J. J. Copeland* (16772 WRT); NANTUCKET: Coskata Pond—*T. Morong* 5, July 15, 1887 (NY); *W. H. Sheldon*, Aug. 6, 1931 (MMA); [coll.?], Aug. 21, 1896 (MMA); Polpis—*Maria Owen*, July, 1879 (NY); Sesachacha Pond—*Dame, Jenks* and *Swan*, July 14, 1880 (NY); *F. S. Collins*, July, 1886 (Y, NY, 7929 WRT, 823 P. B.-A. as *C. crinita* f. *leptosperma*); *L. L. Dame*, Aug. 1886 (NY); *E. P. Bicknell* 11633, Sept. 16, 1899 (NY); Wawinet—*T. F. Allen*, Aug. 30, 1895 (132435 UC, NY, 19782 WRT, RDW; dupl. herb. *T. F. Allen*, as *C. crinita* Waltr., det. *T. F. Allen*); ? Pond—*L. L. Dame*, Aug., 1880 (NY); July 11, 1886 (NY).

7. *Chara delicatula* C. A. Agardh, Syst. Alg., p. 130. 1824. (*nom. illeg.*)⁵
 (Non) *Chara delicatula* Desvaux ex Lois., Not. Fl. France, p. 137. 1810
 (= *C. aspera* (Dethard.) Willdenow, *fide* Migula (1897: 654)).
Chara fragilis var. *delicatula* von Leonhardi, Verhandl. Naturf. Vereins Brünn
 2: 209. 1864.
Chara verrucosa Itzigsohn, Bot. Zeit. 8: 338. 1850, *fide* Robinson (1906: 280).
 References for the region: *Chara fragilis*: Croasdale (1935: 96 [pl. not seen]); var.
delicatula: Owen (1888: 75); *C. delicatula*: Croasdale (1935: 96).

(Plate II, Fig. 5; Plate IV, Fig. 3, 4)

Plant monoecious, 10 cm. high; slightly incrustated with lime; starch bulbils abundant on rhizoids. *Main axes* regularly triplustichous, secondary cortical cells generally somewhat smaller in diameter than the primary cells; spines at cortical nodes reduced to mere papillae. *Branchlets* 8 in a whorl, articulations 7–10, corticated except for terminal 1–2 cells; bracts at sterile nodes inconspicuous. *Stipulodes* apparently haplostephanous, reduced to mere papillae at some nodes. *Gametangia* conjoined at branchlet nodes, but frequently one or other sex appears to be lacking. *Oogonia* 850–1000 μ long by 450–500 μ broad; convolutions 12, very nearly perpendicular to long axis of oogonium; coronula elongate at maturity, 250–270 μ tall, cells generally connivent. *Oospores* 460–500 μ in diameter, black; striae 12–13; membrane opaque. *Antheridia* 410–455 μ in diameter. (Descr. from *R. D. Wood* 2032 (RDW).)

Variations among the local forms are numerous, and result from differences in expression of the vegetative characteristics. In size, some plants reach 25 cm. in height. Other variations appear to be of two distinct orders: (1) a remarkably constant differentiation into forms with two well-developed series of stipulodes (diplostephanous) as in *R. D. Wood* & *Urda K. Wood* (2058 RDW) and forms with one well-developed series (haplostephanous) as in *R. D. Wood* 2032; and (2) less constant differences which appear in random combination. The haplostephanous form is the typical form as described by Groves and Bullock-Webster (1924: 65); whereas the diplostephanous form (Pl. IV, Fig. 4) agrees closely with the description of var. *barbata* (Ganterer) Groves and Bullock-Webster (1924: 68, pl. 44, fig. 9). The features subject to less constant variation are several. In general habit, the plants vary from very attenuate form in *W. R. Taylor* (10171 WRT) to densely compact form in *R. D. Wood* 2000. The number of ecorticated terminal branchlet articulations varies from 3–6 in *R. D. Wood* 2002 to 1–2 in *W. R. Taylor* (10171 WRT). Generally, the penultimate cell of branchlet is not swollen, but it may be rather inflated as in *R. D. Wood* 2022 or very elongate as in *R. D. Wood* 2002. The general trend in expression, as in *C. Braunii*, is toward more attenuate

⁵ The problem of just what is the valid name for this species is one of long standing. The plant has become known as *C. delicatula* C. Ag., but this name is a later homonym of *C. delicatula* Desv. (= *C. aspera*, *fide* Braun; cf. Groves & Bullock-Webster, 1924: 51, 67). Robinson (1906: 280) decided upon *C. verrucosa* Itzigsohn (1850: 338), but it is not known if he saw the type specimen of that plant. There are at least three synonyms in the literature prior to 1850, including *C. pilifera* C. Ag. (1824: xxviii), *C. virgata* Kützinger (1834: 56), and *C. foliolata* Hartman (1820: 378). The problem can be solved only by inspection of the type specimens, a task most easily accomplished by students in the countries in which the critical specimens are to be found.

plants with longer branchlet articulations, shorter bracts, bracteoles, and stipulodes in quiet, deeper water; and compact, tufted, frequently bulbiliferous (with abundant starch bulbils on rhizoids) plants with longer bracts, bracteoles, and stipulodes in shallow littoral.

This species varies widely, and approaches *C. globularis* Thuill. (= *C. fragilis* Desv.) very closely. At one extreme, it is almost identical in vegetative characters to *C. aspera*. Croasdale's (1935) record of *C. fragilis* for Falmouth Heights was probably *C. delicatula*.

Illustrations: Typical form—Groves, H. & J. (1880: pl. 207, fig. 1a); Migula (1897: 753; 1925: 242, fig. 7); Groves & Bullock-Webster (1924: pl. 44, figs. 1-8). Var. *barbata*—Groves & Bullock-Webster (1924: pl. 44, fig. 9).

Exsiccatae: Phyc. Boreali-Amer., No. 1199 as *C. fragilis* subsp. *delicatula*; Char. Amer. Exsicc., No. 22.

Localities: CAPE COD: Chara Haven—*R. D. Wood* 2010, July 7, 1947 (RDW, NY); *R. D. Wood & Urda K. Wood*, Sept. 6, 1947 (2058 RDW); Chara Pond—*M. S. Doty*, June 28, 1947 (2001 RDW, MBL, MSD, ETM); *R. D. Wood* 2002, June 28, 1947 (RDW, NY); Little Pond, Falmouth Heights—*R. D. Wood* 2006, July 7, 1947 (RDW, NY), 2032, Aug. 1, 1947 (RDW); Oyster Pond Annex [?], S.E. of Oyster Pond—*W. R. Taylor*, July 4, 1925 (10172 WRT, MICH); Salt Pond—*W. R. Taylor*, July 15, 1917 (2294 WRT); *Hannah Croasdale*, July 14, 1946 (6614 MSD); *R. D. Wood* 2000, June 28, 1947 (RDW, NY), 2018, July 26, 1947 (RDW), 2024, 2025 (NY, RDW), 2022, 2026, July 30, 1947 (RDW); CUTTYHUNK: Gosnold Pond—*W. R. Taylor*, July 15, 1919 (2931 WRT); Sheep Pond—*W. R. Taylor*, July 9, 1925 (10171 WRT), June 28, 1932 (17012, 17083 WRT, NY), July 3, 1934 (16767, 16766 WRT); *R. D. Wood* 2028, July 31, 1947 (RDW, NY), collected on inland edge of pond, 2029, July 31, 1947 (RDW, NY) collected on seaward edge of pond; NANTUCKET: Sesachacha Pond—*L. L. Dame*, July, 1886 (NY); *F. S. Collins*, July, 1886 (NY); NAUSHON: French Watering Place—*W. R. Taylor*, July 6, 1920 (3105 WRT).

8. *Chara aspera* Willd., Ges. Naturf. Fr. Berlin 3: 298. 1809.

The writer has seen no herbarium specimens which are undisputably of this species for the region, and as such regards the local records as highly questionable. Of the specimens seen, the sterile condition rendered differentiation from *C. delicatula* almost impossible, and recently collected fertile specimens have all proved to be *C. delicatula* Ag. However, during the summer of 1948, Dr. E. A. Palmatier and Dr. V. I. Cheadle collected excellent male and female specimens of *C. aspera* in Rhode Island just across the Massachusetts state line in Ashawonk's Swamp, Little Compton. The chances are good that *C. aspera* should be found in our region in brackish-water ponds.

The best practical distinguishing feature between the American forms of these two species seems to be the fact that *C. aspera* is dioecious, whereas *C. delicatula* is monoecious. A generalized description of *C. aspera* is almost identical to *C. delicatula* in vegetative parts. In *C. aspera*, the female plant has whorls of branchlets which are more spreading than the closely convergent branchlets of the male plants. Antheridia are borne at most of the nodes of fertile branchlets of male plants. Oogonia are borne at the first three (occasionally fourth) fertile nodes of female plants. G. O. Allen (corresp., 1948) stated that normally *C. aspera* has long spine-cells [spines] which are short in *C. delicatula*. The writer has studied the New England specimens, particularly those for Massachusetts and Rhode Island, and concludes that this is helpful but finds that in this region *C. aspera* may be practically devoid of spines; hence, this character of European plants does not appear to hold for our specimens.

Illustrations: Allen (1882b: pl. 21, fig. A, 1-3, 7); Migula (1897: 656, 657); Groves & Bullock-Webster (1924: pl. 39).

Exsiccatae: Phyc. Boreali-Amer., No. 1196; Char. Amer. Exsicc., No. 26, 27.

Localities: none known.

SPECIES EXCLUDED FROM LOCAL FLORA

Chara fragilis Desv. (= *C. globularis* Thuill.); from Chara Pond, Cape Cod, *fide* Croasdale (1935) = ? *Chara delicatula* Ag. (specimen not seen by author).

Nitella batrachosperma (Reich.) Braun (= *N. Nordstedtiana* Groves, H. & J.); from Nantucket, *fide* Owen (1888, determined by T. F. Allen) = *N. Morongii* Allen *emend.* Wood.

Nitella gracilis (Sm.) C. Ag.; from Wood Pond, Golf Pond, Cape Cod, *fide* Croasdale (1935), Nobska Pond, *fide* F. S. Collins (P. B.-A., No. 1195) = *N. Morongii* Allen *emend.* Wood.

Nitella maxceana Allen, *spec. dub.*; from [Maxey's Pond] Nantucket, *fide* T. F. Allen (1896) = *N. Morongii* Allen *emend.* Wood.

Nitella mucronata (Braun) Miquel var. *gracillima* Groves & Bullock-Webster; from Wood Pond, Cape Cod, *fide* Croasdale (1935) = *N. megacarpa* Allen.

TABLE I

	<i>Nitella flexilis</i>	<i>Nitella megacarpa</i>	<i>Nitella Morongii</i>	<i>Nitella transilis</i>	<i>Chara Braunii</i>	<i>Chara canescens</i>	<i>Chara delicatula</i>
Cape Cod							
Ashumet Pond	0	P	0	X	0	0	0
Chara Haven	0	0	0	0	X	0	X
Chara Pond	0	0	0	0	X	PX	X
Desmid Haven	0	0	0	0	X	0	0
Golf Pond	0	0	P	0	0	0	0
Harper Pond	0	0	X	0	P	0	0
Ice House Pond	0	0	0	0	X	0	0
John Pond	0	0	0	P	0	0	0
Leech Pond	0	P	0	0	0	0	0
Little Pond	0	0	0	0	X	P	X
Nobska Pond	0	0	P	0	P	0	0
Oyster Pond	0	0	0	0	X	0	0
Oyster Pond Annex	0	0	0	0	0	0	P
Salt Pond	0	0	0	0	X	X	PX
Sandwich Pond	0	0	0	0	X	0	0
Summerfield, South	0	P	0	0	0	0	0
Weeks Pond	X	X	0	X	X	0	0
Wood Pond (Gansett Pond)	0	P	0	0	0	0	0
Cuttyhunk							
Clubhouse Pond	X	0	0	0	0	0	0
Gosnold Pond	0	0	0	0	P	0	P
Sheep Pond	P	0	0	0	PX	0	PX
Sheep Spring	P	0	0	0	0	0	0
Martha's Vineyard							
Chilmark Pond	X	0	0	0	0	0	0
Tiasquam Dam	X	0	0	0	0	0	0

TABLE I—*Continued*

	Nitella flexilis	Nitella mega- carpa	Nitella Morongii	Nitella transilis	Chara Braunii	Chara canescens	Chara deli- catula
Nantucket							
Cato's Pond	P	0	0	0	0	0	0
Coskata Pond	0	0	0	0	0	P	0
Long Pond	0	0	0	0	P	0	0
New Lane Pond	X	0	0	0	0	0	0
Maxey's Pond	0	0	P	0	0	0	0
Polpis.	P	0	0	0	0	P	0
R. R. Track Pond	P	0	0	0	0	0	0
Sesachacha Ditch	X	0	0	0	0	0	0
Sesachacha Pond	0	0	0	0	P	P	P
Siasconset Pond	0	0	P	0	0	0	0
Wawinet Pond	0	0	0	0	0	P	0
Weweeder Pond	P	0	0	0	P	0	0
Naushon							
French Watering Place	0	0	0	0	P	0	P
Petchett (Peckett) Pond	0	0	X	0	0	0	0
Nonamasset							
South Pond	P	0	0	0	0	0	0
Pasque							
West End Pond (Nitella Pond)	X	0	0	0	X	0	0
? Pond, near West End Pond	0	0	0	0	X	0	0
? Pond, smaller pond near S. W. end of island	0	0	0	0	P	0	0
? Pond, near west end of island	X	0	0	0	0	0	0
Penikese							
Typha Pond	0	0	0	0	X	0	0

LOCALITIES FOR CHARACEAE IN WOODS HOLE REGION

In the above table, all localities are listed from which specimens of Characeae have been seen by the writer. The substantiating specimens are listed in the text under "localities" for each species. Because of the great effect of the hurricane of 1938 on the coastal ponds of the region, the writer has chosen this date as the critical year. Specimens collected prior to 1938 are recorded as "P." Specimens collected since 1938 are recorded as "X." If no collections have been seen by the writer, this is recorded as "0."

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THE BIOLOGICAL BULLETIN

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RADIOACTIVE SODIUM PERMEABILITY AND EXCHANGE IN FROG EGGS

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STATEMENT OF THE PROBLEM

The transfer of ions in both directions across the cell membrane is a matter of major biological importance. While problems involving permeability have led to a vast and prolix literature, only meager attention has centered on the specific distribution of electrolytes within single cells. The limitations of earlier techniques were emphasized by Hastings (1941) in his Harvey lecture on the tissues and body fluids, in which he early recognized the value of radioactive isotopes. The subject of permeability has been recently reviewed by S. C. Brooks (1945). Our interest has centered in the dynamic equilibria of a protoplasmic system, their control by diffusion and the blocking of internal exchange. Use of radioactive tracer techniques has given new precision to the qualitative and quantitative study of small ionic transfers within cellular dimensions. Accordingly, in this paper are given results of an investigation undertaken to determine how much and how fast traced sodium (containing Na^{24}) exchanges with normal sodium (Na^{23}) in a vertebrate egg.

MATERIALS AND METHODS

Ovarian "winter" eggs of the frog, *Rana pipiens*, were chosen because they are single cells, spherical and of large size. Throughout the fall and winter quarters of the year, they are readily available in quantity (without hormone stimulation). In addition to the vitelline membrane, ovarian eggs possess two thin epithelial layers which could be removed successively by fine-pointed forceps. However, in most of the experiments, these membranes were left intact, because of the possibility of injury to the cell surface. Amphibian eggs are ideal for volume measurements. One unique advantage is that they possess an extremely large nucleus, or germinal vesicle, approximately 0.5 mm. in diameter, which may be isolated easily under a dissecting microscope in a Ca-free medium (Duryee, 1937).

Eggs were isolated individually in standard Ringer solution: NaCl, 0.66 g.; KCl, 0.014 g.; CaCl_2 , 0.012 g.; distilled H_2O , 100 ml. All solutions were buffered to pH 7.6 with NaHCO_3 . As described below, Na^{24} was incorporated in experimental solutions as NaCl.

Active healthy frogs were selected. After ovaries of pithed animals were removed as bloodlessly as possible, fresh Ringer solution was poured over them before transfer to small beakers. A small lobe of an ovary was next cut off with iridectomy scissors taking care not to injure the cells. Groups of 20–30 eggs were next transferred to Syracuse dishes where individual eggs were cut apart. All small yellow or transparent eggs were removed and the ovarian wall was trimmed off flush with the theca membranes of each full-size egg. In this way, using a dissecting microscope to insure precise excision, it was possible to obtain from 40 to 60 separate eggs per hour. Average volume was 2.86 mm^3 , based on an average diameter of 1.76 mm. An important step was the reexamination of individual eggs to reject those which might have been nicked or crushed. Aspherical eggs were also discarded along with any less than 1.65 mm. in diameter. Following these procedures, a final screening for uniformity of diameter was made. This consisted of shaking approximately 100 eggs in a flat bottom shallow dish to obtain a single layer with close packing. Any cell not obviously tangent to its six neighbors was discarded.

Eggs were conveniently handled individually with a pipette having an internal diameter just larger than that of the cell itself. In a few experiments where short exposures involving minimum transfer of external radioactive material was essential, each egg was advantageously picked up by the short flap of theca membrane with sharp forceps.

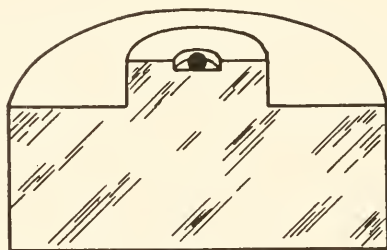


FIGURE 1. Sectional view of plexiglas cup for measuring radioactivity in frog egg. Actual size.

For experiments on dead cells, a method of killing involving minimal change was sought. After rejecting reagents such as alcohol, acetic acid and KCN, we standardized on heating to 65°C . for ten minutes in normal Ringer.

Groups of 10–20 eggs were exposed in small beakers containing 20 ml. of Ringer. Temperature was maintained in water baths controlled to $\pm 0.5^\circ \text{C}$. The presence of water vapor surrounding the beakers kept down evaporation, which by weighing was found to be negligible over periods up to 24 hours.

Upon removing a cell from the radioactive Ringer, it was put through two washes of normal Ringer before transfer to the measuring cup. One or two eggs were first transferred in a vertically held pipette allowing them to sink to the meniscus. By merely touching the surface of the washing fluid, the eggs dropped

from the pipette, thus avoiding transfer of more than one or two cubic millimeters of previous fluid. Washing vials each held 7 ml. It was considered advisable to use a fresh pipette for each successive transfer. Since it was found that Na^{24} could be washed from the cell interiors, the process was reduced to a standard thirty seconds.

For measurement in the Geiger counter, eggs were transferred from the second washing vial to special plexiglas cups (see Fig. 1) again in a vertically held pipette. Not more than 5 mm³ of the second wash fluid was thus transferred with the egg. Tests of this fluid showed negligible radioactivity.

Radioactive solutions

The radioactive Na^{24} was prepared in the cyclotron by bombardment of metallic sodium with 16 m.e.v. deuterons. The metal was dissolved in ethyl alcohol and converted into NaCl with 12 N hydrochloric acid. The mixture was then dried and ignited at red heat to produce a neutral salt free of organic material. Weighed amounts of the radioactive salt were converted into amphibian Ringer solution. To obtain a relation between quantity and radioactivity of Na^{24} , a known aliquot of the Ringer was evaporated to dryness and measured with a Geiger counter. A typical result was as follows: 1 ml. of amphibian Ringer was diluted to 500 ml. A volume of 0.039 ml. of this solution was dried and measured on the counter. Since 1 ml. of the Ringer contained 0.026 g. Na, the aliquot possessed $0.026 \times 1/500 \times 0.039 = 2.03 \times 10^{-7}$ g. Na. This sample gave 1600 counts/min. Thus 1000 counts/min. represented 1.27×10^{-7} g. Na.

For each solution made up with radioactive sodium, controls were made up with identical amounts of normal NaCl. The radioactivity of the solutions was such that the radiation level was less than an amount which would produce 1 r.e.p./min. No visible cytological changes were produced by this level of radiation. In view of the well-known tolerance of frog eggs to much larger dosage of X-rays, it is fair to assume that radiological effects were negligible in our results.

Radio-autographs

A new variant of conventional methods of making radio-autographs has been devised to fit the purpose of recording radioactivity distribution within a single cell. Our technique consisted essentially of quick-freezing in liquid air, followed by sectioning the cell to a known thickness, and then exposing the section in a light-proof cold box to a photographic plate.

Eggs were rapidly rinsed (5 sec.) in two washes of normal Ringer (7 ml. each), oriented in a 1.8 mm. hole in thin calibrated bronze strips (1.5×3.5 cm. and either 80 or 200 μ thick) and plunged into liquid air. The orientation was such that the axis of the egg was parallel to the surface of the strip. It was calculated that freezing of a 1.7 mm. egg at -180°C . would be completed at the center in about 0.2 sec. This time should be compared with the ten minutes necessary for partial equilibrium of ionic diffusion in the egg at normal temperatures. The bronze strip containing the frozen cell was then placed on a previously cooled brass block (see Fig. 2) and both were transferred to the stage of a dissecting microscope. Using a cooled razor blade mounted in a special non-conducting handle, the frozen egg was sliced down to expose the nucleus. Orientation was

thus re-checked to insure that the plane of section was being made parallel to the egg axis. Improperly oriented eggs were discarded. Subsequent slices through the lateral third of the nucleus were made, making the surface even and parallel with the bronze strip. The nucleus always stood out sharply as a white circle in a grey background of frozen cytoplasm. Finally the strip was inverted and the cell was sectioned similarly from the other side. Frozen sections were conveniently temporarily stored in a cold slotted brass holder in an insulated box, cooled with solid CO_2 .

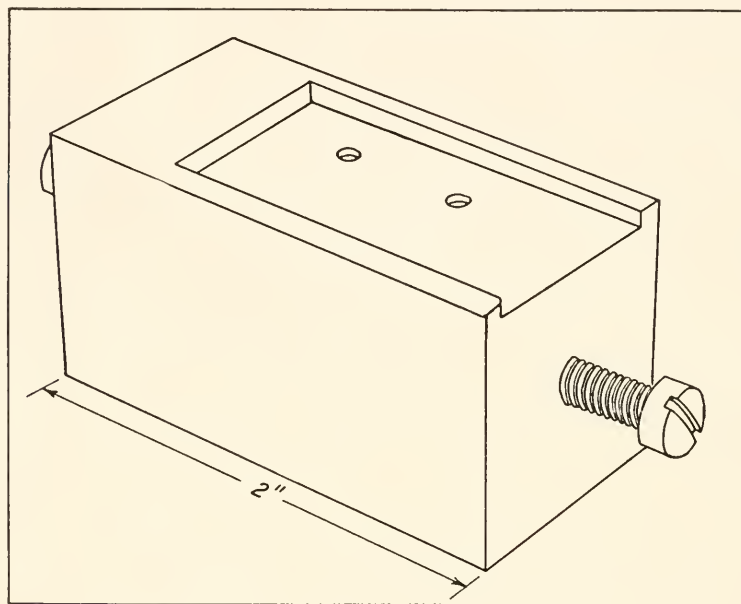


FIGURE 2. Brass cooling block for holding frozen egg and supporting strip during sectioning. Note that both holes corresponding to eggs in holder are displaced laterally from the main axis of the block, so that the strip, when inverted after first sectioning, brings cut surface against flat portion of the block. Screw at each end makes convenient attachment for handle.

Actual exposure of the section to film took place in a well-insulated chest fitted with a copper bottom and copper slide holder. One hundred pounds of dry ice kept the temperature in the slide holder at approximately -40°C . for a week. Medium-contrast lantern slide plates¹ were laid in the slide holder, emulsion side up, and the cold bronze strips with the frozen sections were laid on the plates, being weighted to insure even contact. With the activity of our solutions, it was found necessary to expose from two to twelve hours to provide the desired darkening. Plates were developed in Eastman D-19 developer.

A typical calculation indicates the amounts of radioactivity involved. A section of a frog egg $80\ \mu$ thick contained 2.0×10^{-8} g. of exchanged sodium. This sample

¹ We have learned from Dr. Kenneth Endicott, of the National Institutes of Health, that Ansco non-screen X-ray films are particularly useful where only low intensities of radiation are present.

gave 320 counts/min. on the counter. The actual number of disintegrations occurring in the egg section was approximately 20/sec. An exposure of four hours sufficed to give a good darkening showing a clear differential distribution between nucleus and cytoplasm. This amounts to 288,000 electrons, about half of which went into 0.026 cm² of the plate emulsion.

RESULTS

Sodium content of ovarian eggs

The sodium content of frog eggs is not well known. Bialaszewicz (1929) has given an approximate figure of 42 mg. Na/100 g. wet weight of eggs of *Rana temporaria*. It seemed desirable, therefore, to determine sodium on our experimental animals. For this purpose, both ovaries were removed from six animals. The ovaries were rinsed quickly in distilled water, blotted, weighed, and cautiously ashed over low heat by concentrated HNO₃, followed by concentrated H₂SO₄.

After removal of a small amount of insoluble material, probably CaSO₄, the sodium was determined as (UO₂)₃ZnNa(CH₃COO)₉·6H₂O according to the method of Barber and Kolthoff (as given in Hillebrand and Lundell, 1929). Results obtained from six pairs of ovaries gave a preliminary value of 137 mg. Na per 100 g. wet weight of eggs (Duryee and Abelson, 1947). Further study has shown these results to be too high and that PO₄ interferes with the accuracy of the method. This is at least in part due to the formation of an insoluble uranium-phosphorus compound which tends toward giving high results for the sodium value. Accordingly the procedure was modified. Following wet ashing, excess acid was driven off, the residue dissolved in distilled water, and the solution made alkaline with NH₄OH. A 25 per cent barium acetate solution was added until no further precipitate formed, followed by a few drops excess. The mixture was allowed to stand for half an hour and then centrifuged. The precipitate was washed twice with distilled water and the washings added to the first supernatant. The solution was evaporated to dryness, the residue dissolved in 1 ml. of distilled water, and the sodium precipitated with the uranyl zinc acetate reagent. The following results were obtained from six pairs of ovaries:

Wt. of ovaries (grams)	mg. Na	mg. Na/g. ovary
8.1	6.8	0.84
9.3	7.40	0.80
9.0	7.4	0.82
8.5	7.0	0.82
7.0	5.9	0.80
8.4	7.1	0.84
Average		0.82 mg. Na

There remained the question of how well the sodium content of the whole ovary approximates that of the eggs. Since the connective tissue, blood vessels and peritoneum formed only a minor fraction of the ovary, it seemed that the error introduced by neglecting this factor would be small. A sodium determination on separated eggs gave 0.80 mg. Na/g. wet weight. Eggs averaged 1.76 mm. in diameter with a volume of 2.86 mm³, a density of 1.10, and a calculated sodium content of 25.8×10^{-7} g. Na per egg.

Water content of eggs

In analyzing the results, it was found desirable to measure the water content of the eggs. Accordingly four determinations were made—two with separated eggs and two with ovaries. Excess moisture was blotted up with filter paper. The eggs were weighed and then dried to constant weight at 120° C. for one hour. A water content of 52 per cent was found. This corresponded to 1.63 mg. of water for our standard 1.76 mm. diameter egg.

An interesting observation gave evidence on the water content of the nucleus. In six sets of eggs which were frozen whole, the half sections, when thawed and dried, showed a negative mold of the nucleus with only a flake remaining. This indicates the relatively higher water content of the nucleus above that of the yolk-cytoplasm.

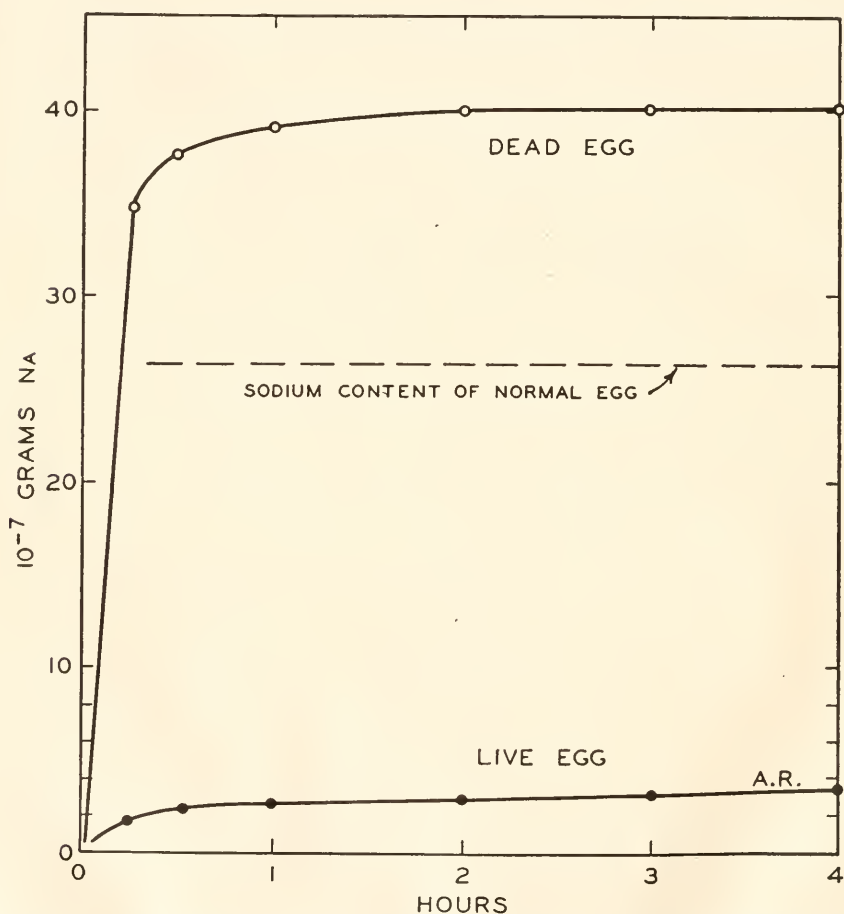


FIGURE 3. Comparison of uptake of Na^{24} by live and dead eggs in amphibian Ringer. Average value of sodium content of single egg, as determined by chemical analysis, shown by dotted line. Each point represents average measurements on ten separate cells.

Sodium exchange

In the course of the research a total of twenty experiments were performed involving 400 individual eggs. The experiments gave a reproducible set of values with a probable variation of plus or minus ten per cent. A comparison of the exchange of sodium in live and dead eggs is shown in Figure 3. Each point on the curve represents an average of ten measurements. In the case of live eggs, the following features are to be noted: Initially there was a rapid penetration of the traced sodium into the egg. Within an hour, however, the amount of the traced substance within the cell ceased to increase. The level reached amounts to 12 per cent of the total sodium content of the egg. By testing samples of cytoplasm and later by making radio-autographs, it was shown that the traced sodium was principally inside the egg.

With dead eggs (Fig. 3) the exchange of sodium was extremely rapid. The amount of traced sodium which could be found in the egg rose above the total sodium content of the live cell as determined chemically. This entrance of extra sodium can be related to at least two facts: We have observed a loss of potassium from the cell on death. The second fact is that the sodium content of amphibian Ringer for a volume equal to that of the frog egg is 78.4×10^{-7} g., or more significantly, the sodium present in a volume of Ringer equal to that of the cell's water content is 42.4×10^{-7} g. Na.

To study further the nature of the sodium uptake process, eggs were first exposed to radioactive Ringer, washed and then immersed in an inactive Ringer solution. The results are shown in Figure 4. During the first hour, a typical curve was obtained for penetration of the traced sodium. The downward curve was obtained during exposure to inactive Ringer. The time required for the traced sodium to leave the cell was about the same as the time involved in the initial penetration. Chemical analysis showed that the total sodium content of the egg did not change during these experiments. When dead eggs containing tracer sodium are exposed to inactive Ringer, the traced sodium likewise leaves the egg very quickly.

Since calcium is thought to be important in permeability relationships, experiments were designed to investigate the effect of this ion on sodium exchange. Three solutions were made up with tracer sodium: The first, an ordinary Ringer solution; the second, with calcium not present; and a third, with double the usual calcium content. When eggs were exposed to these solutions and measured as before, the curves shown in Figure 5 were obtained. These curves show that calcium only slightly influenced that fraction of the sodium (12 per cent) which is exchangeable. After several hours, however, lack of calcium resulted in an increased exchange of the sodium.

A series of experiments was made to determine the effect of temperature on exchange of sodium. Experiments were conducted at 9° C., 20° C., and 30° C. While the speed of penetration of sodium was somewhat faster at the higher temperature, the level reached after an hour was almost identical in all cases. After three hours approximately 6×10^{-7} g. Na was found per cell, while the low temperature values were essentially those of the controls at 20° C. It is apparent, therefore, that sodium exchange is not highly dependent on cellular metabolism.

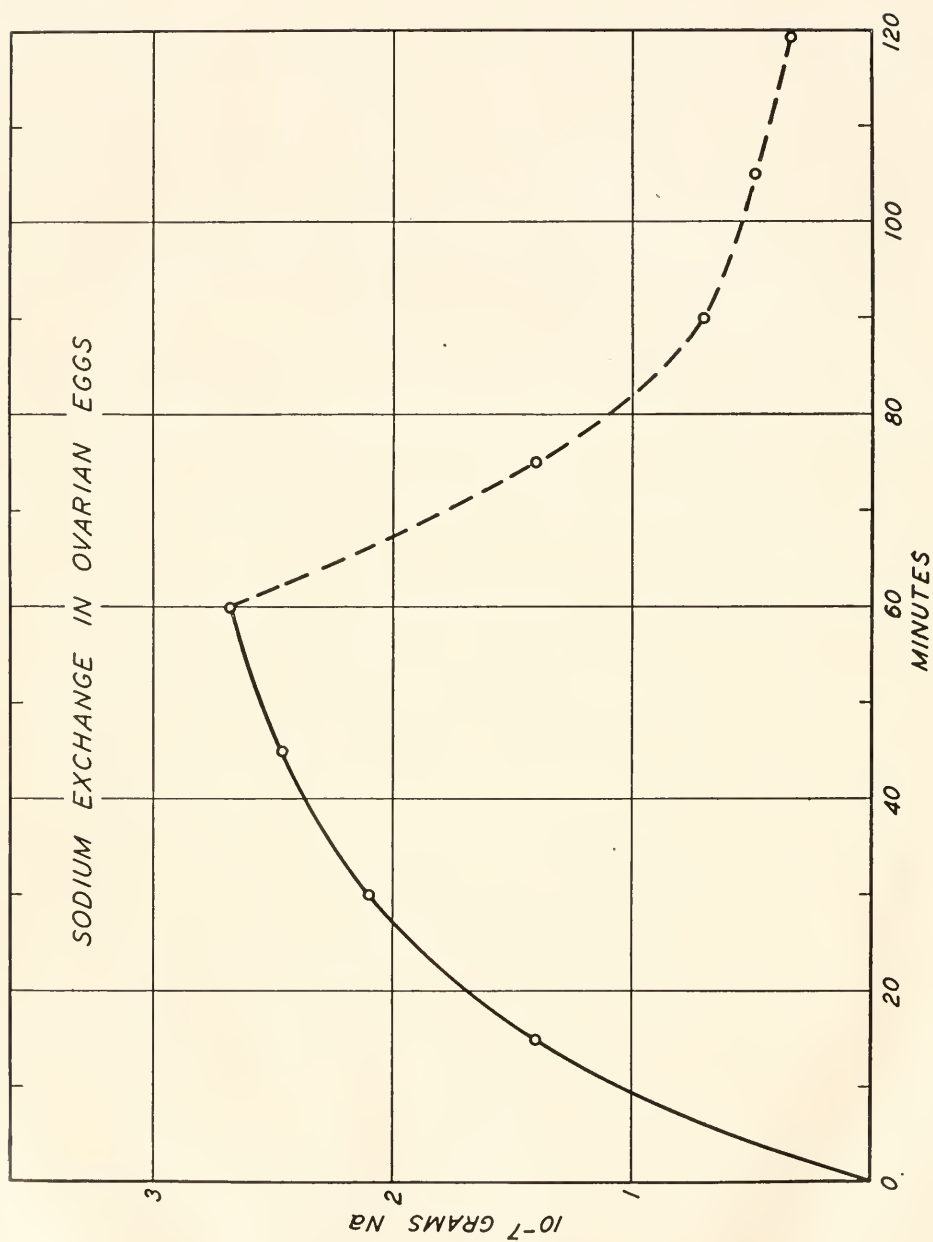


FIGURE 4. Comparison of rate of uptake of Na^{24} in a radioactive Ringer solution with the rate of "washing-out" in a normal Ringer medium.

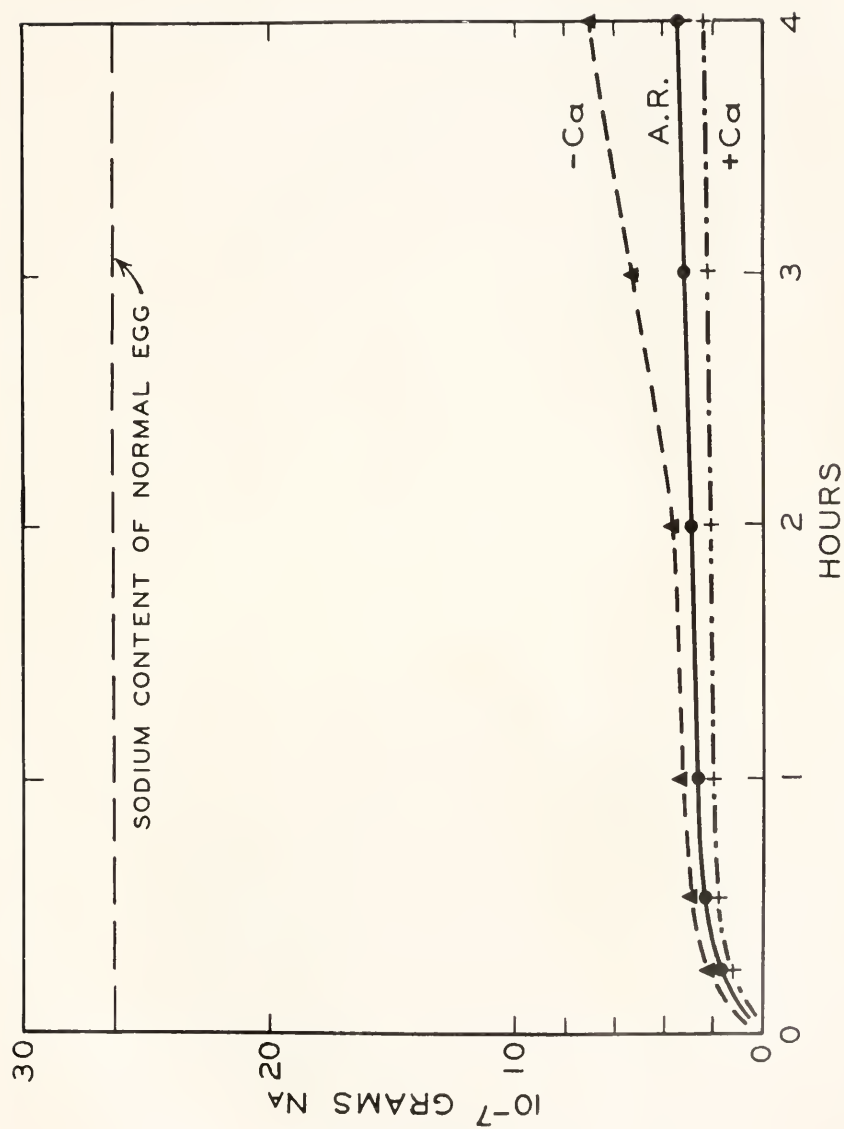


FIGURE 5. The influence of calcium on the rates of sodium exchange in single cells. Calcium-free Ringer ($-Ca$) ---; normal amphibian Ringer (A.R.) solid line; Ringer with double calcium content ($+Ca$) -.-.-.

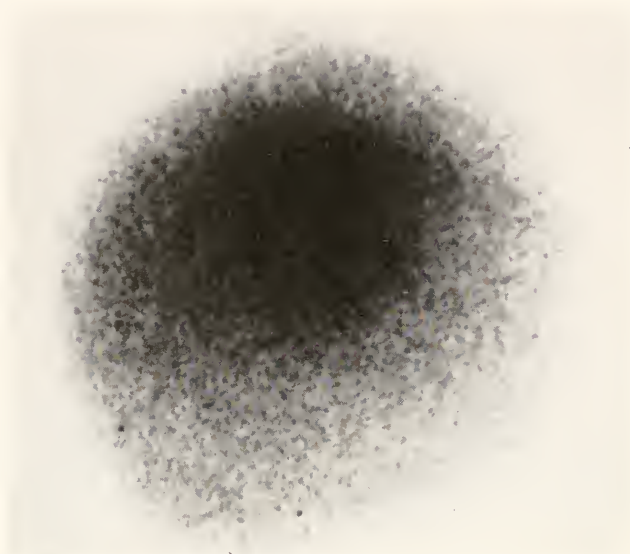


FIGURE 6. Radio-autograph of ovarian egg exposed to Na^{24} for thirty minutes. Cell was frozen in liquid air and sectioned parallel to main axis. Dark portion in upper third corresponds to size and position of the cell nucleus.

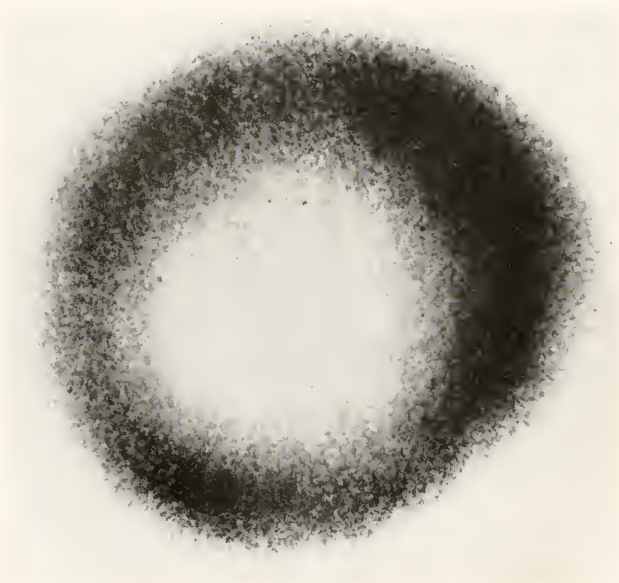


FIGURE 7. Radio-autograph of dead ovarian egg exposed to Na^{24} for five minutes. Width of "ring" is proportional to diffusion rate of sodium inside the cell.

The question of where the sodium goes inside a cell seemed of fundamental importance. Many attempts were made to answer this question with but little success. It was of course possible to isolate single nuclei from exposed eggs. Such nuclei were washed twice in calcium-free nuclear medium to remove outside "cytoplasmic" sodium and were then placed individually on plastic cups for measurement with the Geiger counter. The counts per nucleus were always significantly higher than background, but showed disappointing variation. It was concluded that the washing process introduced an uncontrollable variable. Consequently, we turned to the radio-autograph technique. In Figure 6 is shown an enlargement of a typical radio-autograph of a frog egg frozen in liquid air and sectioned meridianally. Of 47 autographs of single cells exposed to Na^{24} for more than 30 minutes, all showed denser silver particles in the nuclear area than in the cytoplasm, when the plane of section was through the germinal vesicle. In those autographs of eggs sectioned to one side of the nucleus, a uniform density was found throughout the endoplasmic area.

In the analysis of sodium diffusion rates inside the cell, advantage was taken of the radio-autographic records of penetration. In Figure 7 is shown an enlargement of a radio-autograph obtained by exposure of a dead egg to radioactive Ringer for five minutes followed by washing, freezing, and sectioning. When live eggs were exposed to the active Ringer, for five minutes, followed by standard technique, a similar ring-shaped autograph was also obtained with the density of the darkening much less.

DISCUSSION

To serve as a basis of comparison, a calculation² has been made of the time required for diffusion processes to exchange sodium in an element of fluid the size

² An exact calculation of diffusion in an object similar to the frog egg would be practically impossible. First, there is the inhomogeneity of nucleus and cytoplasm. Second, is the fact that only a part of the sodium in the egg exchanges. One important factor in the calculation is that these experiments trace the behavior of Na^{24} in a medium under conditions where there is no net transport of sodium or chloride ions. Therefore, in calculating the diffusion of Na^{24} in Ringer solution, one should use the self-diffusion coefficient of Na^+ rather than that of NaCl . Fortunately, the self-diffusion of Na has been measured in sodium chloride solutions by Jehle (1938) and in sodium iodide solutions by Adamson (1947). In the experiments the concentration of salt is the same on both sides of a diaphragm, and a very small amount of radioactive sodium is added to one of the solutions. From the rate of appearance of tracer sodium in the second solution the self-diffusion coefficient can be measured. The value observed was 1.2×10^{-5} $\text{cm}^2/\text{sec.}$ in 0.113 M NaCl solution, and 1.23×10^{-5} $\text{cm}^2/\text{sec.}$ in 0.113 M sodium iodide solutions.

A calculation giving useful information regarding diffusion into a sphere can be made by employing formulas presented by Barrer (1941). The formulas have been applied to "Ionic Exchange Absorption Processes" by G. E. Boyd et al. (1947). In the case of a completely permeable sphere initially free of the diffusion ion, the time required for the solute to reach half of its equilibrium value is given by

$$Q/Q_{\infty} = 1/2 = 1 - 6/\pi^2 \sum_{n=1}^{\infty} 1/n^2 \exp. (-D\pi^2 n^2 t/r^2)$$

where Q_{∞} is the equilibrium value of the quantity of traced sodium, Q is the value at any time t , D is the diffusion coefficient of Na taken here as 1.23×10^{-5} $\text{cm}^2/\text{sec.}$, and r the radius is 0.088 cm. By a method of successive approximations the above equation is satisfied when $D\pi^2 t/r^2 = 0.3$ and $t = 19$ seconds.

of a frog egg. Assuming no retardation at the surface of the object and a diffusion coefficient of 1.23×10^{-5} cm²/sec. internally, the time required for half of the sodium to exchange is 19 seconds. In the case of the actual egg, the time required for half of the exchangeable sodium to exchange is 15 minutes. The exchange of sodium in the egg may be slower for at least two reasons: delay in passing through the cell membrane, and the existence of a low diffusion coefficient within the egg. The fact that we have obtained "ring" radio-autographs after exposure of five minutes is explainable only on the basis that the movement of sodium in the egg is much slower than in Ringer solution, and that the membrane has little limiting effect on the exchange.

On the assumption that the membrane presents no barrier, one can calculate a value for the diffusion coefficient within the egg. Thus, for $Q/Q^\infty = 1/2$, $D\pi^2t/r^2 = 0.3$, and $D = 2.6 \times 10^{-7}$ cm²/sec.

From the curves given in Figures 3 and 5, it is clear that only 12 per cent of the sodium in the ovarian frog egg is readily available for exchange. Part of the remaining sodium can be exchanged over a period of many hours. Since the factors which govern this slow exchange are wholly within the egg itself, some form of internal blocking must occur. Speculation concerning the mechanism is still unwarranted. We believe that the finding that 12 per cent of the sodium behaves differently from the remainder is of considerable importance to others who are performing tracer experiments. In this experiment, the behavior of the traced substance was not characteristic of the behavior of all the sodium of the cell. Upon the other hand, it would appear that the difficulty raised might be turned into an advantage. One has a technique for studying the various degrees of binding and the chemical activity of cellular components.

It is of special interest that sodium could be traced into the germinal vesicles. Our experiments thus show that the nuclear membrane is permeable to sodium. They also show that sodium is normally present in a cell nucleus. It is especially significant that the concentration of this cation in the nucleus is at least double that of the cytoplasm. The relatively large amount of active sodium found in the nucleus may be connected with the fact that the nucleus has a much higher percentage of water than does the yolk cytoplasm.

Our data on the increased permeability of dead cells to Na²⁴ offer a basis for explaining some of the empirical observations of Quimby (1947). Working with second and third degree burns she was able to show that the more severely damaged tissues took up much greater amounts of tagged sodium. These results must be due to the presence of dead cells. Furthermore, she found that addition of hypertonic saline materially aided recovery. It is clear, therefore, that in both sets of observations a fundamental factor is operating. This epitomizes, in the case of sodium, what many physiologists have long believed to be a basic diagnostic character of life—namely, that a living cell can discriminate between ions both quantitatively and qualitatively in its extracellular environment and can build specific internal gradients and unequal distributions, which a dead cell certainly cannot do. As we have shown in the case of the amphibian egg, permeability to radioactive sodium can be used as an indication of life or death in a cell.

SUMMARY

1. The ovarian egg of the frog *Rana pipiens* is freely permeable to Na^{24} .
2. At least two different types of binding limit the internal diffusibility of sodium within the egg. Only 12 per cent of the normal sodium is readily exchangeable. The remainder exchanges very slowly.
3. The implications of finding non-equilibration of such a simple ion as sodium are presented.
4. A new technique for making radio-autographs of single cells shows that after half an hour sodium is distributed almost uniformly throughout the cytoplasm.
5. Calculations based on rate of exchange of sodium into the egg plus radio-autograph evidence give a value of $2.6 \times 10^{-7} \text{ cm}^2/\text{sec.}$ for the diffusion coefficient of sodium within the egg.
6. At equilibrium the nucleus possesses approximately twice as much traced sodium per unit volume as the cytoplasm.
7. By direct chemical analysis the sodium content of frog eggs was found to be 0.082 per cent of the wet weight.

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STUDIES IN THE REGULATION OF BLOOD-SUGAR CONCENTRATION IN CRUSTACEANS. I. NORMAL VALUES AND EXPERIMENTAL HYPERGLYCEMIA IN *LIBINIA EMARGINATA*

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INTRODUCTION

Studies of blood-sugar concentrations of invertebrates were, until relatively recently, confined for the most part to defining the range of glycemic values in different species. Stimulated by investigations of the effects of insulin on blood-sugar concentrations in mammals, these early studies among invertebrates demonstrated that considerable variations existed in the amount of glucose in the blood, even within a single species. The reviews of Beutler (1939) and of Benazziz-Lentati (1941) summarize many of these observations.

The wide range of glycemic concentrations in crustaceans reported by different investigators, who at times studied the same species, was soon recognized to be due, in part, to the different analytical methods employed and, perhaps even to a greater extent, to the varied physiological states of the animals at the time blood samples were taken for analysis. This latter possibility led to observations on animals maintained under more critical laboratory conditions and to studies of factors that influenced the amount of sugar in the blood.

Thus, where Hemmingsen (1924a) had found an increase in blood sugar in *Astacus* after feeding, Kisch (1929) reported a decrease in *Carcinus maenas* during starvation. These results were confirmed both by Stott (1932) and by Florkin (1936) for the same species of *Carcinus*. On the other hand, Roche and Dumazert (1935) found that the blood glucose of *Cancer pagurus* starved for one month did not differ significantly in concentration from that of freshly captured individuals. Asphyxiation was reported by Stott (1932) to cause marked hyperglycemia in *C. pagurus*, *Portunus puber*, and *Carcinus maenas*; this observation was confirmed by Roche and Dumazert (1935) on *Cancer pagurus*. Stott (1932) also observed a high concentration of glucose in the blood of newly-molted crustaceans (within a few hours of ecdysis) compared with animals before the molt.

Hemmingsen's studies (1924b) on the crayfish *Astacus* led him to believe a regulatory mechanism was present for maintaining a constant level of blood sugar. The basis for this view was his observation that samples of concentrated glucose solution when injected into *Astacus* disappeared from the blood stream too rapidly to have been oxidized to CO₂ during the experimental period, and yet no glucose was excreted in detectable amount into the water in which the injected animals

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were maintained. These results Hemmingsen regarded as evidence for a hypoglycemic regulatory mechanism.

In addition, a number of pharmacological substances had been reported as being effective in inducing hyperglycemia in crustaceans. The controversy in this branch of the general problem lay not so much in the interpretation of such hyperglycemias when they occurred, but whether these substances really induced hyperglycemia. Thus, Medvédeva (1936) reported that injection of adrenalin in *Potamobius* (*Astacus*) caused hyperglycemia, but that injection of insulin was without any definite effect. Roche and Dumazert (1935), on the other hand, reported that neither adrenalin nor insulin had any appreciable effect on the blood-sugar concentration of *Cancer pagurus*. Kalmus and Waldes (1936) stated that not only adrenalin and insulin, but also such non-specific substances as hydroquinone and sodium chloride solution, effected marked hyperglycemias when injected into crayfish. Florkin and Duchateau (1939), using more carefully controlled procedures, reported that insulin had no effect while adrenalin produced a hyperglycemia in the crayfish, thus confirming the observations of Medvédeva.

These scattered observations, controversial though they may have been, indicated the possible existence of a hyperglycemic mechanism, just as Hemmingsen's studies had indicated the possibility of a hypoglycemic mechanism. The first indication of a definite anatomical structure which might be involved in regulating sugar metabolism in crustaceans was made by Abramowitz, Hisaw and Papandrea (1944). These authors found that injection into *Callinectes sapidus* of aqueous extracts of crustacean eyestalks increased the concentration of blood sugar within an hour. More specific localization of the source of this diabetogenic factor was demonstrated by the preparation and injection of extracts prepared from the sinus glands of Hanström that had been removed from eyestalks. The injection of such extracts resulted in a marked hyperglycemia amounting to nearly four times the normal basal concentration of blood glucose. When extracts, prepared from the remainder of the eyestalks from which the sinus glands of Hanström had been previously removed, were injected, they were practically without hyperglycemic effect. These investigators' complementary experiments, which consisted of removing the sinus glands by ablation of both eyestalks, to determine whether hypoglycemia would ensue, gave negative results; in fact, over a period of seven days after eyestalk removal, there was an anomalous, slight increase in concentration of blood sugar.

Our own studies were undertaken to define in greater detail the nature of such glycemic changes under experimental conditions and to investigate the physiology of the regulatory processes. This is the first report in detail of our investigations, some of which have appeared in abstract form (Kleinholz, 1948; Kleinholz and Little, 1948; Kleinholz and Havel, 1948).

MATERIALS AND METHODS

The animals used in this study were the marine spider crab, *Libinia emarginata*. A large stock of animals was maintained by the laboratory collectors in a live-car. Other than the occasional placing of a freshly killed fish into the live-car, no regular feeding of these stock animals was undertaken. When groups of animals were removed for use in the laboratory, they were starved for three days before

blood samples were taken, to insure a basal level of blood-sugar concentration, and were not otherwise fed except where indicated. Such experimental animals were marked for identification by painting serial numbers with lacquer on the dorsal surfaces of the carapace of each; the crabs were then placed in individual containers, similarly numbered, through which a stream of sea water circulated. It was hoped that the hyperglycemic effect of crowding reported by Abramowitz et al (1944) would be reduced or obviated by such isolation. Only male individuals were used in this study.

Tuberculin hypodermic syringes of 1 ml. capacity, graduated in hundredths of a milliliter, were used for taking blood samples. Each syringe was calibrated to deliver 0.5 ml. by weighing the volume of water delivered between the 0.60 ml. and 0.10 ml. marks on the barrel of the syringe. Blood for analysis was taken from the sinuses of the walking legs, the arthroidal membrane between the base of the leg and the body being first wiped dry with filter paper or absorbent cotton. The syringe was filled slightly beyond the 0.60 ml. mark and, after withdrawing the needle from the sinus, was emptied to this mark, the excess droplet of blood being removed by touching the tip of the needle to filter paper. A 0.5 ml. sample of blood could thus be delivered into tubes containing the deproteinizing mixture. To avoid injury to the arthroidal membrane that would ensue from the repeated bleeding of the same individual, samples were taken from different legs on both sides of the animal. In all of the experiments reported here, blood samples from control and from experimental animals were taken in the daytime, in most cases in the forenoon. The possibility of a diurnal variation in concentration of blood sugar was thus avoided.

It was found most convenient to work with groups of six *Libinia* at a time. In most instances blood samples were taken from the individuals of a group before a particular treatment, and then again after the experimental treatment, each crab thus serving as its own control. The control blood samples and the experimental blood samples were carried through the analytical procedure simultaneously and received comparable handling. The method for determining the amount of blood glucose was that described by Miller and Van Slyke (1936). It is reported that this method, when used with mammalian blood, gives "true" blood-sugar values, which do not include non-fermentable reducing substances. We have found a significant amount of non-fermentable reducing substance present in *Libinia* blood, so that in our hands the method must be considered as expressing *total reducing substances* as glucose equivalents. The procedure was essentially as described by Miller and Van Slyke. The dilute ceric sulfate for the titration was prepared fresh daily from the stock solution.

Two blanks, consisting of the reagents used in the glucose determination, were used with each set of blood samples. These blanks required about 0.15–0.25 ml. of the dilute ceric sulfate to reach the same end-point obtained in the titration of the blood samples. This wide range was due to the preparation of a second lot of stock reagent solutions during the course of the work. For the first set of stock solutions the blanks varied from 0.18–0.25 ml., the average for 38 blanks being 0.22 ml.; with the second set of stock solutions the blanks varied from 0.13–0.18 ml., the average for 20 blanks being 0.15 ml. The average for the total 58 blanks was 0.195 ml. of the dilute ceric sulfate.

The accuracy of the Miller and Van Slyke method in our hands was tested by determining the amount of glucose in prepared solutions of known concentration. These concentrations ranged from 16–200 mg. of glucose per 100 ml. of solution. The average percentage of error for 13 such determinations was ± 3.9 per cent.

Where *Libinia* without eyestalks were used to determine the effect of absence of the sinus glands on the blood-sugar level, bilateral eyestalk ablation was done with the aid of fine dissecting scissors. Bleeding from the cut surface was very slight and ceased upon the formation of a blood clot in the orbit.

The distinction between total reducing substances and non-fermentable reducing substances in the blood was made by fermenting one of two blood samples with a 10 per cent suspension of Fleischmann's yeast. The yeast was prepared by suspension in distilled water, centrifuging, and pouring off the supernatant. After three such washings, the final 10 per cent suspension was kept in the refrigerator until used. In the fermentation, 3.5 ml. of the yeast suspension and the 0.5 ml. blood sample were mixed and allowed to remain at 22° C. for one hour, after which the mixture was centrifuged and the supernatant poured off into a second tube containing acid cadmium sulfate. The yeast and blood residue was similarly washed and centrifuged three times with 1 ml. portions of distilled water, the supernatants each time being added to the first one. A second 0.5 ml. blood sample taken from the same animal had been prepared for the routine analysis. Both blood samples were carried through the analytical procedure simultaneously, using adequate blanks (washings of a 3.5 ml. aliquot of yeast suspension) for the fermented samples.

The reliability of this method of fermenting glucose in blood was tested with samples from six *Libinia*. To a 0.5 ml. portion of blood from each animal was added 0.5 ml. of solution containing 5.06 mg. of glucose (thus equivalent to adding 1012 mg. per cent of glucose to the blood sample) and 3.5 ml. of the 10 per cent yeast suspension. The blood samples were fermented and treated as described above. The glucose-equivalent in reducing substances present in these samples ranged from 6.3–11.1 mg. per cent, with an average of 8.9 mg. per cent, showing practically complete fermentation of the added glucose.

OBSERVATIONS

A. Normal and eyestalkless animals

The studies of Abramowitz, Hisaw and Papandrea (1944) pointed to the sinus glands as being mediators in the hyperglycemic response following injection of prepared extracts. Their attempts to observe whether removal of this gland (by ablation of both eyestalks) resulted in hypoglycemia yielded paradoxical results, a gradual hyperglycemia being observed in such animals over a period of seven days.

Our own observations made over a longer period in a comparable series of experimental animals, do not confirm the latter results of these investigators. Of eighteen animals brought into the laboratory at the same time, the eyestalks of twelve were ablated, while the remaining six served as normal control animals. The animals of the control group were isolated in individual containers; six of the operated *Libinia* were designated as Group A and were similarly isolated, while the remaining six operated animals, constituting Group B, were placed in a com-

mon tank. These animals were not fed during the time the experiment was in progress. Beginning on the morning of the third day after eyestalk removal, blood samples were taken from the individuals of Group A and of the control group; on the following morning, the fourth day after ES removal, samples were taken for analysis from individuals of Group B. By alternating in this fashion and taking blood samples every third day, the observations were extended over a period of twenty-six days. The results which are shown in Table I are the averages and the standard deviations for the six animals constituting each group.

TABLE I

Comparison of the blood-sugar concentrations in two groups of Libinia without eyestalks, with that of a normal control group. The figures are the averages for the 6 crabs of each group and their standard deviations.

Days after eyestalk removal	Blood-sugar concentration in mg.-per cent		
	Group A	Group B	Control
3	10.1 $\pm$ 2.5	—	6.9 $\pm$ 1.2
4	—	11.1 $\pm$ 2.1	—
6	12.0 $\pm$ 3.1	—	9.9 $\pm$ 1.5
7	—	9.0 $\pm$ 2.5	—
10	10.2 $\pm$ 3.4	—	7.4 $\pm$ 1.3
11	—	9.3 $\pm$ 3.6	—
17	10.2 $\pm$ 4.3	—	7.3 $\pm$ 1.8
18	—	9.6 $\pm$ 3.7	—
24	10.1 $\pm$ 3.2	—	8.7 $\pm$ 1.7
26	—	7.9 $\pm$ 2.6	—

As might be expected, there were variations in the concentration of blood sugar not only among the individuals of a group, but also in the same individual at the different intervals when blood was taken for analysis. These variations are probably due to a combination of actual fluctuations in glucose concentration and of slight artifacts in the analytical procedure. The relatively low average of the blood-sugar concentration in the control group on the third day, compared with later averages, is probably to be explained on this basis, for the average glycemic values determined for three different normal groups, which had been starved three, six, and ten days, were, respectively, 7.9, 8.3 and 9.1 mg. per cent. We conclude from the data of Table I that removal of the sinus glands has no marked effect on the basal level of the blood-sugar concentration. The hyperglycemia reported by Abramowitz et al. after eyestalk removal in *Callinectes* could not be confirmed with *Libinia*.

B. Total reducing substances and true blood sugar

It has been known that the blood of mammals contains, in addition to glucose, other reducing substances which, in the analytical methods currently employed, may contribute significantly to the total value obtained as "apparent" glucose. For an accurate measure of the amount of glucose in a blood sample the supple-

mentary use of a yeast fermentation method along with the conventional determination permits the distinction to be made between total reducing substances or apparent blood glucose, and non-fermentable reducing substances; the difference between the two such determinations is then considered to represent the fermentable glucose.

The application of methods devised for the analysis of mammalian blood to that of invertebrates would require similar supplemental yeast fermentation methods, since little is known about the presence or the nature of non-fermentable reducing substances in blood of the latter group. Such yeast fermentation analyses of the blood of *Libinia*, using the procedure described under "Methods," were conducted in parallel with samples taken at the same time for the determination of total reducing substances. The results for experimental and control animals are arranged in Table II.

TABLE II

True blood-sugar concentrations in groups of eyestalkless and of normal Libinia. Each group consisted of 6 animals. TRS, total reducing substances; NFRS, non-fermentable reducing substances; TBS, true blood sugar. Figures are averages for the animals of a group and the standard deviations from the mean.

Animals and condition	Concentration in mg. per 100 ml. blood		
	TRS	NFRS	TBS
Group A, starved 24 days	10.1 $\pm$ 3.2	7.4 $\pm$ 0.8	2.7
Group B, starved 26 days	7.9 $\pm$ 2.6	5.0 $\pm$ 1.3	2.9
Controls, starved 24 days	8.7 $\pm$ 1.7	6.8 $\pm$ 1.7	1.9
Normal, starved 6 days	8.3 $\pm$ 1.3	6.3 $\pm$ 0.7	2.0

The data shown in this table represent the averages for four groups of *Libinia*, each group consisting of six animals. Three of these groups consisted of individuals whose blood analyses for total reducing substances had been made at intervals over nearly four weeks, as shown in Table I. The animals of Group A, Group B, and the control group are described in the text above and in the preceding table. On the twenty-fourth and twenty-sixth days when final analyses were being made on these three groups, additional samples were taken at the same time for determination of the non-fermentable reducing substances after the blood sample had been mixed with yeast suspension and fermented. The fourth group of Table II consisted of normal animals which had been starved six days, in comparison with the twenty-four days of starvation undergone by the control group to the eyestalkless condition. The figures which are given for concentration of true blood sugar are averages for the six individuals of a group, representing the differences between the average concentrations of total reducing substances present in one set of samples and the average amounts of non-fermentable reducing substances found in similar samples after they had been fermented by the yeast suspension.

In all groups the amount of true blood sugar is quite low. There appears to be no difference in glycemic level between normal animals starved for a short period (6 days) and those starved for an appreciably longer time (24 days). At

first glance the slightly higher level of true blood sugar in the groups of eyestalkless individuals as compared with that in the normal control animals might seem to indicate an alteration in glucose metabolism as a result of eyestalk removal, but in view of the small number of animals involved in the experimental groups and the comparatively high standard deviations of the averages for each group, it is doubted that these differences from the controls can be regarded as significant.

C. Hyperglycemia as a result of injection of eyestalk extract

The hyperglycemic effects obtained by Abramowitz, Hisaw and Papandrea (1944) in *Callinectes* upon injection of eyestalk extracts showed a rough agreement between dosage and the increment of the resulting hyperglycemia. But since their determinations were in terms of total reducing substances, with no distinction being made between fermentable and non-fermentable components, closer examination was made of these components of the total reducing substances at the same time that we tried to confirm their observations.

Blood samples from a group of six *Libinia* from which both eyestalks had been ablated were analyzed on the first and fifteenth days after eyestalk removal. At these times the average concentrations of total reducing substances were respectively 7.1 ± 1.5 mg. per cent and 6.3 ± 1.6 mg. per cent, confirming previous observations made in Table I that no significant change follows in the glycemic level of animals from which both eyestalks have been removed. On the morning of the seventeenth day after eyestalk ablation, each of the crabs was injected with 0.1 ml. of extract prepared from the eyestalks of *Libinia*, so as to receive the equivalent of one eyestalk. One hour after the injection, blood samples were taken from each animal for determination of both the total reducing substances and the non-fermentable reducing substances. Five of the six injected *Libinia* showed striking increments in total reducing substance in the blood, the concentrations after injection being from twice to nearly five times those obtained before injection; in the sixth animal the amount of total reducing substance after injection was about 60 per cent greater than before the injection. The average value for total reducing substance for all six animals was 17.3 ± 6.4 mg. per cent. The average for non-fermentable reducing substances of the post-injection samples after yeast treatment was 4.5 ± 0.4 mg. per cent; average fermentable blood sugar after injection was therefore 12.8 mg. per cent. Yeast fermentations were not made on blood samples taken on the first and fifteenth days after eyestalk removal, but if the average value for true blood sugar is assumed to be comparable to those shown in Table II, then the average increase in fermentable blood sugar after injection of eyestalk extract is well over 400 per cent. [We are thus able to confirm the observation of Abramowitz et al. that injection of crustacean eyestalk extract induces a marked hyperglycemia in crustaceans, and to show, furthermore, that this increase is apparently a fermentable sugar.

D. Hyperglycemia as a result of asphyxia

Stott (1932) had reported a large increase in blood sugar of crustaceans which had been kept for ten hours in containers of sea water that had been tightly covered. This change he attributed to asphyxia due to the decrease in oxygen content of the

water during the period of the experiment, because when an adequate air supply was again made available to the animals by removing the cover, the blood-sugar concentration returned to the normal level. Roche and Dumazert (1935) confirmed this observation by reporting that removing animals from sea water and keeping them in air for 30–60 minutes resulted in a marked hyperglycemia. Both studies reported this hyperglycemia as a direct observation, with no attempt to investigate in further detail the mechanism of this response.

Similar results were obtained by us with *Libinia*, and a possible mechanism for what we shall call the hyperglycemia of asphyxia was indicated by further study. In these experiments, groups of six normal and six eyestalkless animals were employed. To obtain partial asphyxia during which the animals could be kept under observation, the method of Roche and Dumazert was used: removing the animals from sea water and keeping them in air for 60 minutes, and then removing a blood sample from each for analysis and for comparison with samples before asphyxia. The results of these experiments are shown in Table III.

TABLE III

Effect of asphyxia on the blood-sugar concentration of normal animals and animals without sinus gland

Animal group	Eyestalk condition	Days starved	Concentration in mg.-per cent	
			Before asphyxia	After asphyxia
Group B Nos. 13–18	ES off 31 days	31	(7.9 $\pm$ 2.6)*	7.0 $\pm$ 2.8
Controls Nos. 19–24	Normal	31	(8.7 $\pm$ 1.7)*	16.1 $\pm$ 8.3
Nos. 31–36	ES off 1 day	18	7.1 $\pm$ 1.5	6.0 $\pm$ 1.1
Nos. 25–30	Normal	19	7.3 $\pm$ 1.0	22.0 $\pm$ 12.2

* See Table I.

The first two groups of crabs tested consisted of eyestalkless individuals which had been under observation for several weeks (the animals constituting Group B of Table I), and a similar number of normal *Libinia* which had been their controls. Both groups were removed from sea water and placed in individual finger-bowls in air. After one hour of such exposure, blood samples were taken for analysis (at this time the animals were limp and showed a marked loss of muscular tone; following their return to sea water recovery was rapid). No blood samples were taken in this experiment directly before the asphyxiating experience, the glycemic values which had been determined at regular intervals for the preceding twenty-six days being considered sufficient to serve as a standard. As can be seen from Table III, the effect of this asphyxia was different in the two groups, the crabs without eyestalks showing no appreciable change in their average concentration of blood sugar, while the group of normal animals showed a marked increase in the glycemic average for the group.

The experiment was then repeated with two additional groups of similar animals. This time a blood sample was removed before subjecting the animals to asphyxia, and the second sample was taken immediately after the 60 minutes of asphyxia. The results were similar to those obtained previously; the normal animals (with eyestalks) showed a marked hyperglycemia, the average concentration being three times the pre-asphyxia level, while the animals without eyestalks showed no significant change from the glycemic level before asphyxia. These results show that the observed hyperglycemia is dependent upon the intact eyestalk and indicate the possibility that the response may be mediated by the sinus gland. The results of more exact studies, in which sinus glands were removed from otherwise intact eyestalks, to define the mechanism of the hyperglycemic response, will be reported later.

E. Alimentary hyperglycemia

A number of investigators have reported the effects of feeding and inanition upon blood-sugar levels in crustaceans. The studies of Henningsen (1924a) and Stott (1932) showed that feeding resulted in a rise in blood-sugar concentration. Stott had found that in a group of starved *Carcinus maenas*, the glycemic level ranged between 5–8 mg. per cent. When such animals were fed mussels, the blood sugar rose to values of 20 mg. per cent or more over a period of several hours; about fourteen hours after such feeding, the level of blood sugar returned to a concentration of approximately 5 mg. per cent.

In view of the part played by the eyestalk and sinus gland in mediating the hyperglycemia resulting from asphyxia, as described in the preceding section, it was thought advisable to determine whether alimentary hyperglycemia was similarly regulated. Seven *Libinia* from which both eyestalks had been removed three days previously, and which had been starved for three days, were isolated in individual containers. The average glycemic value immediately before feeding was 9.1 ± 2.8 mg. per cent. Each animal was then supplied with 5–10 grams of the visceral mass of *Venus mercenaria*, which was devoured within fifteen minutes. Blood samples taken three hours after this feeding showed a marked rise in sugar content in each of the seven animals, the average for the group after feeding being 18.3 ± 4.7 mg. per cent. The results therefore indicate that alimentary hyperglycemia is not mediated by the sinus glands in the eyestalks.

SUMMARY

1. Removal of the sinus glands by eyestalk ablation in unfed *Libinia emarginata* has no significant effect on the blood-sugar concentration when compared with similarly unfed controls.

2. Values for true blood sugar, as distinguished from total reducing substances, were determined after yeast fermentation of blood samples. In starved animals the concentration of total reducing substances is between 8–9 mg. per cent; that of non-fermentable reducing substances, 6–7 mg. per cent; that for true blood sugar is therefore about 2 mg. per cent.

3. Injection of eyestalk extract increases the concentration of total reducing substances in the blood. This increase is in the fermentable component, amounting

to over 400 per cent of that in the uninjected animal, and therefore probably represents a true hyperglycemia.

4. Asphyxia also causes hyperglycemia, the total reducing substances in blood samples being two to three times the concentration preceding asphyxia.

5. Removal of the sinus gland by eyestalk ablation prevents the appearance of the hyperglycemia of asphyxia. The sinus gland may be a mediator in certain hyperglycemic responses of crustaceans, but does not seem to be concerned in alimentary hyperglycemia.

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OVARIAN INHIBITION BY A SINUS-GLAND PRINCIPLE IN THE FIDDLER CRAB

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The action of the sinus gland in inhibiting ovarian development was first demonstrated by Panouse (1943, 1944, 1946) working with females of the shrimp, *Leander serratus*. In these animals, amputation of both eyestalks or bilateral removal of the sinus glands resulted in a great acceleration of ovarian growth, maturation of the oöcytes, and even laying of mature eggs, during a period when these structures are normally quiescent or just beginning the normal growth phase. Implantation of sinus glands into abdomens of destalked animals resulted in an inhibition of ovarian development. Similar results following eyestalk removal were obtained with the crayfish *Cambarus immunis* by Brown and Jones (1947).

The following experiments were performed upon the fiddler crab, *Uca pugilator*, to ascertain whether this phenomenon of ovarian inhibition by a blood-borne principle from the sinus glands also obtained in the division, Brachyura, of the Crustacea.

MATERIALS AND METHODS

The animals used in the experiments were females of *Uca pugilator* collected near Woods Hole, Massachusetts, on July 10, 1948. The carapace widths ranged from 15 to 20 mm. at the widest point. They were kept in the laboratory at room temperature (about 25° C.) in individual containers each holding sea water to a depth of a quarter of an inch. The water was changed daily. The animals were not fed during the course of the investigation.

Removal of eyestalks was accomplished by amputation at their bases and the wounds were allowed to close spontaneously by clotting of the blood which welled slowly from them.

Sinus glands were obtained from donor animals for the purpose of implanting according to the following procedure. Eyestalks were removed as above, placed in sea water, and the contents of the eyestalks exposed by a dorsal splitting of the chitinous sheath. The sinus gland, a discrete bluish organ, was dissected free of surrounding tissue and drawn into the lumen of a 25 gauge needle by means of a tuberculin syringe. The gland, plus a minute quantity of sea water, was injected into the ventral hemocoel of the recipient animal's abdomen. The chitinous membrane of this region of the body is transparent and thus it is possible to see the actual extrusion of the contents of the needle. The dissections and implantations were accomplished with the aid of a dissecting microscope.

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In order to observe the influences of the above procedures, experimental and normal control animals were autopsied as they died, or were sacrificed for dissection at six-day intervals. The carapace and hypodermis were removed, and the ovary, an H-shaped organ lying over the hepatopancreas and just below the hypodermis, was dissected out in sea water and placed in a tared watchglass for weighing. Excess moisture was removed with filter paper, and fresh weights were taken.

RESULTS

On July 11, 1948, ten normal animals were sacrificed and the ovaries removed. Eight of these ovaries were found to be in an immature state: the oöcytes were very small and the organs as a whole were slender and of a light yellow-pink color. The other two ovaries contained somewhat larger oöcytes and the color of the organ was a deep shade of pink. The average of the ten ovarian weights was 12.6 mg., the extremes being 6.8 mg. and 18.8 mg. respectively.

On July 12, 120 animals of nearly uniform size were selected and divided into three lots. Eyestalks were removed from two lots of forty of them. Two days later, one sinus gland was implanted into each of one lot of forty of the eyestalkless animals using the technique described earlier. The implants were then repeated every fifth day for the duration of the experiment.

TABLE I

Number of specimens, and ranges and averages of ovarian fresh weights in milligrams

Days	Destalked			Destalked, receiving sinus gland implants			Controls		
	No. spec.	Range	Av.	No. spec.	Range	Av.	No. spec.	Range	Av.
1-6	6	11.8-46.2	24.0	14	7.2-45.2	20.6	6	9.2-16.2	13.5
7-12	4	17.9-61.2	32.6	10	6.4-67.6	24.7	4	6.6-13.1	10.1
13-18	5	32.6-54.9	42.2	4	12.5-35.8	21.0	4	4.2-34.2	18.4
19-24	11	23.0-165.6	54.7	5	8.8-32.3	19.5	11	5.8-23.6	13.9
25-30	6	33.8-160.4	66.4	4	15.8-22.3	17.9	6	4.4-19.4	11.0

In Table I are summarized the ovarian weights of the three groups of animals (destalked, destalked and receiving sinus gland implants, and normal controls) which died or were sacrificed during five succeeding six-day periods. Each value obtained represents the average of data from 4 to 14 animals. It will be observed from Table I that over the thirty-day period the average ovarian fresh weights of the destalked animals increased approximately linearly with time from an original 12.6 mg. to 66.4 mg., a more than five-fold increase. The ovarian weights of destalked animals receiving sinus-gland implants showed an initial rise, with an approximate level being maintained at values somewhat higher (a total average of 7 mg.) than those of the control animals.

Other changes observed in the ovaries of the destalked animals, in addition to the gross size alteration, were gradual increases in oöcyte diameter and a shift of their color from the previously mentioned pinkish-yellow to a deep purple-red. The color change became most striking as the ovary attained a weight of approximately 15–18 mg.

In general, the color and size of oöcytes of the destalked animals receiving sinus-gland implants were found to lie somewhere between the extremes offered by the destalked and control animals.

During the course of the experiment, only 7 of the 31 control animals were found at autopsy to have ovaries in the apparently mature condition typical of the destalked ones. On the other hand, after the first six-day period, in no case did any of the latter group contain oöcytes presenting an immature appearance, either in size or in color.

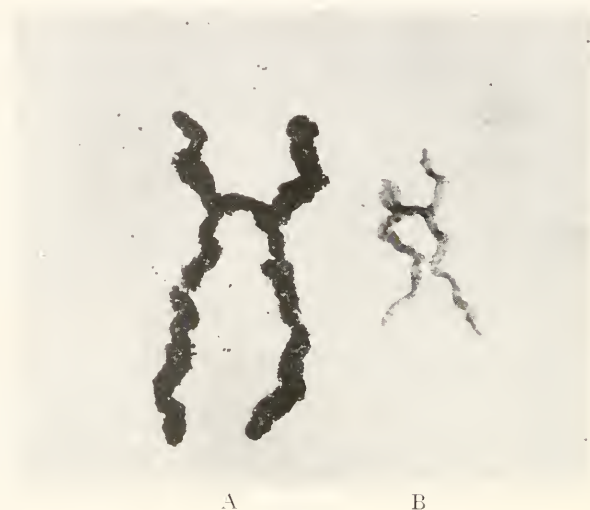


FIGURE 1. Ovaries of two fiddler crabs removed September 1, 1948: A, from an animal destalked one month earlier; B, from a normal animal.

Figure 1 is a photograph of two ovaries removed from animals of the same carapace width—17 mm. The animal from which ovary A was removed was destalked on July 30, 1948, and sacrificed for dissection on September 1, 1948. The animal from which ovary B was removed was a normal control maintained under identical laboratory conditions during the same period and sacrificed on the same day. These two organs are typical of those removed from destalked and normal animals, respectively, during the course of the experiment. The approximate wet weights are: ovary A, 80 mg.; ovary B, 10 mg.

It is also of interest to note that during the time that the investigation was in progress, five females which had been deprived of eyestalks and received no sinus-gland tissue laid mature eggs, and one female, also eyestalkless, which had received one sinus gland implant, did likewise. In none of these cases were the

eggs fastened to the pleopods of the animal as normally occurs. No eggs were laid by any of the control animals.

DISCUSSION

It seems apparent from the foregoing results that the sinus gland in *Uca*, as in *Leander* and *Cambarus*, is the source of an ovary-inhibiting principle which, when absent, allows for a period of ovarian growth and development even at a time when no such gonadal activity would otherwise be manifested.

Implantation of the quantity of sinus-gland tissue utilized in this work into the abdomens of destalked animals tends to suppress the gonadal growth, but allows the ovary to be maintained at a stage somewhat more mature than that characteristic of the normal animals in possession of both sinus glands.

There is no indication from these experiments whether the principle from the sinus glands inhibits the ovary directly or serves to inhibit the production of a gonad-stimulating principle normally produced elsewhere in the body. On the latter hypothesis one possible explanation of the ovarian growth during the first six-day period in the gland-implanted animals is that during the two days elapsing between eyestalk (sinus gland) removal and the first implant, the blood titer of the inhibitor dropped to such a point that a gonad-promoting principle was permitted to be liberated into the blood. The first implant might be presumed to inhibit further production of the stimulating principle but not counteract the action of this factor already present.

An explanation based upon an hypothesis of a direct inhibition of ovarian growth is as follows: During the two days which elapsed between amputation of the eyestalks and the initial implantation of the sinus-gland tissue, there was a drop in titer of the inhibitory substance to an ineffectual level which permitted nearly as rapid growth in these ovaries as occurred in the destalked animals which received no implanted sinus glands. There may also be a delay in the production of an inhibiting concentration by the implants.

There is a suggestion in Table I that sinus-gland implants in the eyestalkless animals not only are able to inhibit growth in the partially developed ovaries, but may even effect a reduction in their size.

SUMMARY

1. Removal of the eyestalks of adult females of *Uca pugilator* results in a period of rapid ovarian growth in which the increase in fresh weight of the gonad is approximately five-fold in a thirty-day period.

2. The period of ovarian growth is characterized by increase in oöcyte diameter and a color change from light pink to a deep purple-red.

3. Implantation of sinus-gland tissue into the abdomens of destalked females serves to inhibit to a large degree this rapid growth.

4. Six of the animals which had been deprived of their eyestalks laid mature eggs during the course of the experiment; none of the controls did so. Eggs produced by the experimental animals failed to become attached to the pleopods.

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PIPERAZINE DIHYDROCHLORIDE AND GLYCYLGLYCINE AS NON-TOXIC BUFFERS IN DISTILLED WATER AND IN SEA WATER ^{1, 2}

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A wide selection of buffers is necessary in biological work, since it is often desirable to repeat a particular experiment with a different buffer. Piperazine dihydrochloride and glycylglycine are crystalline, non-volatile, very soluble solids readily obtainable in pure form. Piperazine is relatively non-toxic to man (Hanzlik, 1917) and to rats (Dieke, Allen, and Richter, 1947) and has been used as an apparently non-toxic buffer by certain biologists at our suggestion (Cornman, 1940, 1941; Evans, Beams, and Smith, 1941). The buffer merits of glycylglycine in sea water have been previously pointed out by Tyler and Horowitz (1937). This relatively non-toxic material is a normal constituent of many proteins. A wide-range buffer is simply and accurately prepared from only these two substances and sodium hydroxide. Used in sea water there is no observed precipitation of salts until a pH of 9.9 is reached. The commonly used phosphate buffer precipitates calcium and magnesium phosphate from sea water at a much lower pH, thus disturbing the salt balance and adding uncertainty to conclusions from experiments. The shortcomings of many of the buffers in common use have recently been mentioned by Gomori (1946). We have not used the new buffers suggested by him and cannot compare his buffers with ours, except to point out that our buffers have a wider range.

For special cases where it is desired to have no inorganic ions in a buffer, it is possible to obtain buffers from a pH of 7.0 to 11.0 by titrating glycylglycine with the free base of piperazine. However, we are presenting no data on this subject.

In this paper we present a table indicating the preparation of several buffers, using piperazine dihydrochloride, glycylglycine and equimolecular mixtures of the two substances in distilled water and in sea water. We also present pK_1 and pK_2 values of piperazine dihydrochloride.

EXPERIMENTAL

The piperazine was purchased from the Eastman Kodak Co. in the form of the hexahydrate. Because the free base of piperazine absorbs carbon dioxide and moisture from the air, it was converted into the stable dihydrochloride (Sieber, 1890) before use.

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² We are indebted to Dr. W. Mansfield Clark for certain suggestions relative to the manuscript, and to H. G. Smith for help with the experiments.

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Piperazine dihydrochloride is prepared by dissolving 50 g. of piperazine hexahydrate in 100 ml. of 95 per cent ethanol, and adding slowly 100 ml. of concentrated hydrochloric acid. Heat is evolved. As the mixture cools, crystals of the dihydrochloride hydrate are formed. The mixture is cooled in an ice bath and is filtered. The crystals are washed several times with cold ethanol, and are air-dried. The material is ready for use after it has been dried at 100° C. for eight hours. The yield is 33 g. Anhydrous piperazine dihydrochloride is slightly hygroscopic.

Analytically pure glycylglycine was purchased from the Amino Acid Manufactures of the University of California at Los Angeles and was used without further purification. The material was dried at 100° C. for six hours just prior to use. Glycylglycine is not appreciably hygroscopic.

The pH measurements were made at 25° C. (± 0.2) with a Leeds and Northrup potentiometer-electrometer No. 7660, equipped with Leeds and Northrup glass dip electrode Std. 1199-12 made of Corning 015 glass, and a reference saturated calomel half-cell electrode Std. 1199-13 with a potassium chloride capillary salt bridge. Before and after each titration the electrode was checked against "standard acetate," for which the pH value of 4.64 was taken (MacInnes, Belcher, and Shedlovsky, 1938). As is the practice in standardizing buffers, the liquid junction potentials were neglected.

The sodium ion error for the higher pH values was corrected by using the following equation adapted from Powney and Jordan (1937) to fit the sodium ion errors found experimentally when our glass electrode was calibrated with the hydrogen electrode.

$$\text{Log } \Delta\text{pH} = 0.50 \text{ pH} - 5.86 + 0.46 \log [\text{Na}^+]$$

The possible error in these readings increases with increasing alkalinity, but below a pH of 9.0, the accuracy was within the limits of ± 0.02 .

Although stock solutions of piperazine dihydrochloride and glycylglycine may be prepared, it is preferable to prepare the solutions fresh, since on long standing glycylglycine may undergo hydrolysis and piperazine dihydrochloride might form toxic products (Greenbaum, 1937).

Solutions of piperazine and glycylglycine and equimolecular mixtures of the two were titrated with standardized sodium hydroxide and numerous readings were taken. From these readings a table for the preparation of buffers was made (Table I).

Several pH determinations were made with the glass electrode and the hydrogen electrode on solutions which were equimolecular with respect to piperazine dihydrochloride and the monohydrochloride (pK_1') and on solutions which were equimolecular with respect to the monohydrochloride and free piperazine (pK_2') (Table II).

DISCUSSION

We have used the available data in Table II in making approximate calculations of the ionization exponents of piperazine at infinite dilution, since this has not been previously reported. Using the standard Debye-Hückel equation for moderately dilute solutions, we have found values for piperazine dihydrochloride for pK_1 of

TABLE I

Table for Preparation of Buffers at 25° C.

- (1) 0.1591 g. piperazine dihydrochloride diluted to 100 ml. with distilled water to which is added 0.1000 N sodium hydroxide as indicated below in column (1).
- (2) 1.591 g. piperazine dihydrochloride diluted to 100 ml. with distilled water to which is added 1.000 N sodium hydroxide.
- (3) 15.91 g. piperazine dihydrochloride diluted to 100 ml. with distilled water to which is added 1.000 N sodium hydroxide.
- (4) 0.1321 g. glycyglycine diluted to 100 ml. with distilled water to which is added 0.1000 N sodium hydroxide.
- (5) 0.1591 g. piperazine dihydrochloride plus 0.1321 g. glycyglycine diluted to 100 ml. with distilled water to which is added 1.000 N sodium hydroxide.
- (6) 0.1591 g. piperazine dihydrochloride diluted to 100 ml. with filtered sea water (pH 8.0) to which is added 1.000 N sodium hydroxide.
- (7) 0.1591 g. piperazine dihydrochloride plus 0.1321 g. glycyglycine diluted to 100 ml. with filtered sea water (pH 8.0) to which is added 1.000 N sodium hydroxide.

Buffer pH	ml. of NaOH to be added to above solutions						
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
4.4	0.66				0.007		
4.6	1.11	0.78			0.054		
4.8	1.71	1.18	7.0		0.120		
5.0	2.47	1.72	11.1		0.202		
5.2	3.38	2.48	17.4		0.302		
5.4	4.44	3.46	25.8		0.419	0.088	0.025
5.6	5.65	4.55	35.5		0.538	0.200	0.140
5.8	6.74	5.73	46.6		0.651	0.353	0.262
6.0	7.62	6.80	58.1		0.745	0.493	0.399
6.2	8.35	7.67	69.0		0.827	0.615	0.524
6.4	8.89	8.35	78.0		0.899	0.718	0.655
6.6	9.27	8.89	85.0		0.953	0.797	0.765
6.8		9.23	90.0		0.999	0.854	0.852
7.0			93.6	0.57	1.045	0.895	0.933
7.2				0.88	1.091	0.923	1.017
7.4				1.37	1.152	0.947	1.105
7.6				2.07	1.231	0.965	1.195
7.8				2.94	1.329	0.983	1.305
8.0				3.93	1.445	1.001	1.435
8.2				5.03	1.571	1.027	1.573
8.4				6.14	1.697	1.065	1.695
8.6	10.66			7.17	1.825	1.113	1.821
8.8	11.00	10.83	107.9	8.01	1.950	1.176	1.949
9.0	11.51	11.23	112.1	8.65	2.064	1.262	2.070
9.2	12.19	11.85	117.9	9.07	2.185	1.362	2.194
9.4	13.09	12.70	125.2	9.40	2.313	1.488	2.338
9.6	14.18	13.75	134.6		2.451	1.642	2.520
9.8	15.39	14.90	145.6		2.587	1.865	2.750
10.0	16.60	16.05	156.6		2.717		
10.2	17.68	17.09	166.6		2.832		
10.4	18.60	17.98	176.2		2.937		
10.6	19.35	18.74	184.4		3.042		
10.8		19.39	190.5		3.153		
11.0		19.94	195.2				

TABLE II
*Relations Between pK' and Ionic Strength for Piperazine
 Dihydrochloride at 25° C.*

Ionic strength	pK_1'	Ionic strength	pK_2'
0.0100	5.44	0.0174	9.74
0.0238	5.49	0.0519	9.78
0.100	5.56	0.174	9.82
0.238	5.68	0.571	9.87
1.00	5.79	0.800	9.88
1.67	5.86		

5.32 and pK_2 of 9.70. Bredig (1894) found a second ionization constant of 6.4×10^{-5} at 25° C., but failed to report a first ionization constant. Kolthoff (1925, 1925) reported a pK_1' of 4.05 and a pK_2' of 8.34 for piperazine at 15° C. Since the temperature he used is different from that used in the present experiment, the results are not comparable. A search of the literature reveals no other reports of the ionization exponents of piperazine.

On the other hand, because of interest in amphoteric electrolytes and in dipeptides, numerous studies of the K_a , K_b , pK_1' , pK_2' , pK_1 , and pK_2 values of glycylglycine have been made (Euler, 1907; Dernby, 1916, 1917; Eckweiler et al., 1921; Harris, 1923; Levene et al., 1924; Täufel and Wagner, 1927; Branch and Miyamoto, 1930; Mitchell and Greenstein, 1930; Fromageot and Watremez, 1930; Stiasny and Scotti, 1930; Greenstein, 1933; Johnson and Peterson, 1935; Neuberger, 1937; Konikov, 1938; Carr and Shutt, 1939; Glasstone and Hammel, 1941; Smith and Smith, 1942). Included above are numerous titration curves for glycylglycine in different media, but no actual tables for the preparation of buffers have been previously reported.

SUMMARY

The advantages of piperazine dihydrochloride and glycylglycine as buffers include the low toxicity, the lack of volatility, the solubility, the availability of the pure products, the convenience and accuracy of buffer preparation, and the lack of precipitation of calcium and magnesium salts from sea water below a pH of 9.9.

Table I indicates the preparation of solutions of known pH involving piperazine dihydrochloride, glycylglycine, and mixtures of these two in distilled water and in sea water at 25° C. Because the determinations were not made with the hydrogen electrode this table must be considered as being susceptible to small error, especially on the alkaline side of pH 9.

Table II shows the pK_1' and pK_2' values for piperazine dihydrochloride for several ionic strengths. Using the Debye-Hückel equation, the extrapolated pK values of piperazine at infinite dilution were found to be 5.32 for pK_1 and 9.70 for pK_2 .

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CHROMATOPHOROTROPINS IN THE CENTRAL NERVOUS ORGANS OF THE CRAB, *HEMIGRAPSUS OREGONENSIS*

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INTRODUCTION

Early work on the humoral control of crustacean chromatophores has demonstrated that the sinus gland is the most important source of chromatophorotropic substances. Investigations leading to this conclusion are discussed in Brown's review (1944, pp. 130-134). Later work by Brown (1946) and Brown and Saigh (1946) has shown that most crustacean central nervous systems also possess at least two chromatophorotropic principles, one causing all portions of the body of *Crago* except the telson and uropods to become pale (*Crago* body-lightening hormone, CBLH), and a second (*Crago*-darkening hormone, CDH) darkening the telson and uropods, and in the absence of CBLH, the body also. CDH, however, was absent in the *Brachyura* studied.

This paper reports experiments undertaken for the purpose of determining whether or not chromatophorotropins are present in the central nervous system of the Pacific coast shore crab, *Hemigrapsus oregonensis*. Most attention was given to the optic ganglia, but some experiments were performed to test the brain and thoracic ganglia. I wish to thank Dr. R. I. Smith for his many helpful suggestions and criticisms.

MATERIALS AND METHODS

Only male crabs were used. Their eyestalks were ligated with number 80 cotton thread on successive days before injections were made. After the melanophores were completely punctate, the crabs were injected with *Carcinides* perfusion fluid (Pantin, 1934), and those whose melanophores responded at all were not used in subsequent experiments.

Organs from which extracts were made were rinsed in several changes of *Carcinides* perfusion fluid to remove any blood adhering to them and transferred to a roughened depression slide containing a drop or two taken from a measured quantity of perfusion fluid. Here they were torn apart, crushed, and triturated with fine forceps under a dissecting microscope, care being taken to ensure as complete extraction as possible. The extract was then transferred with an eyedropper to the measured quantity of perfusion fluid, and the depression slide was rinsed with this perfusion fluid several times. The extract was boiled for a few seconds, allowed to settle, and the supernatant fluid was used for injections. Sterile needles and syringes were used for all injections.

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To record the responses of the melanophores, an arbitrary index of four stages was used, from complete concentration of the pigment (stage 1) to complete dispersion (stage 4). The somewhat opaque and pigmented cuticle of *Hemigrapsus oregonensis*, especially in the larger crabs, obscures the chromatophores over much of the body, and observations were therefore made on the arthrodial membranes at the bases of the legs.

In preliminary experiments it was found that the melanophores of *H. oregonensis* responded to injections of sufficiently strong extracts of muscle and gill, as well as to weak egg albumin solutions (Fig. 2). Such preparations, particularly the latter, certainly do not contain chromatophorotropins, and the melanophore response in such cases probably is part of a rather generalized stimulation resulting from the introduction of foreign substances into the hemolymph. Since it was essential to eliminate such responses when testing extracts of central nervous organs for chromatophorotropins, this was done by making up the extracts from comparable volumes of tissue, small enough so that extracts of them would not contain sufficient protein or other unknown non-humoral material to affect the chromatophores. In this way it was intended to distinguish specific chromatophorotropic effects from the non-specific effects resulting from injection of large tissue masses. In order to make up such extracts it was necessary to know the amounts of tissue in the different organs extracted. Since the organs, especially the sinus glands, were too small to weigh on an analytical balance, measurements were made of their volumes using a procedure suggested by the method of Weil and Pantin (1931) for measuring volume changes in the turbellarian, *Gunda ulvae*. The organ was carefully dissected out, placed in the ruled area of a hemacytometer counting chamber and flattened under the cover glass. An enlarged (1 mm. = 2 in.) outline of the organ was drawn on a piece of paper containing a copy of the ruled area of the hemacytometer; this was traced onto a piece of medium weight drawing paper, cut out, and weighed. By comparing this weight with that of a similarly enlarged square millimeter (0.1 cu. mm.) the volume of the organ could be roughly determined. Table I gives the results of these measurements. All crabs used had a carapace width of 1.7 cm. The numbers are the weights of the paper cutouts in milligrams.

These measurements are admittedly crude, most of the error being due to dissection. They do, however, give some idea of the relative size of the organs involved. The dilution factors are selected values, based on the relative volumes by which extracts of the different organs were diluted to give approximately equal volumes of tissue in the same amounts of extract. For organs other than those listed in Table I (brain, leg nerve, etc.) the volume was measured by the preceding method, and the extract was diluted accordingly.

In preparing extracts, the size of the crab from which the organ was extracted was considered. It was assumed that the size of the sinus gland and other organs varies directly with the weight of the crab; thus, for example, the sinus gland from a crab 1.9 cm. wide (3.0 g.) would be twice the volume of that from a crab 1.5 cm. wide (1.5 g.).

The amount of extract injected was always 5 per cent of the body weight, assuming a specific gravity of 1.00 for the extract. To obviate weighing each crab, a large number of crabs were weighed and the weight plotted against the

TABLE I
Comparative size of sinus gland and optic ganglia

	Sinus gland	Medulla terminalis	Medulla interna	Medulla externa	Lamina ganglionaris	1 mm. ² paper
	19	607	308	311	207	377
	14	767	284	293	282	364
	16	501	272	341	250	355
	18	579	249	250	198	365
	12	550	236	259	157	376
	14	350	210	279		374
av.	15.5	559	260	289	219	369
$\frac{\text{av.}}{3690} = \text{mm.}^3$	0.004	0.151	0.070	0.078	0.059	0.100
Relative volume	1	36.1	16.8	18.7	14.17	
Dilution factor	1	30	15	15	12	

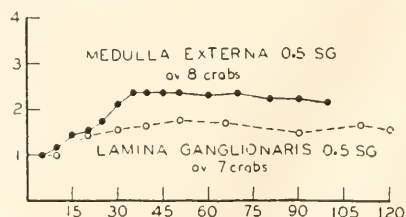
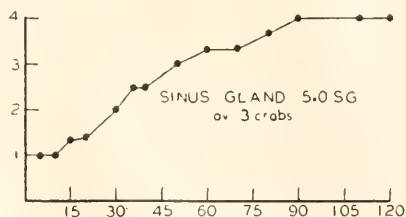
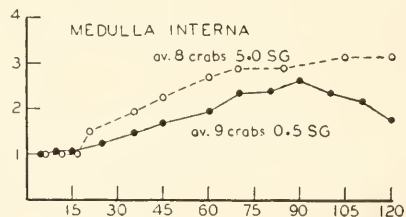
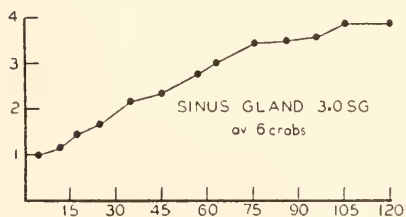
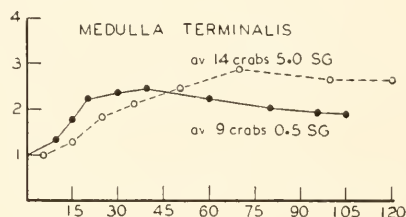
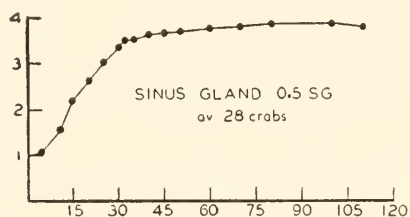


FIGURE 1. Responses of *Hemigrapsus melanophores* to injections of extracts of various organs. Abscissae: time (minutes) after injection. Ordinates: degree of dispersion of melanin (1 = complete concentration; 4 = complete dispersion).

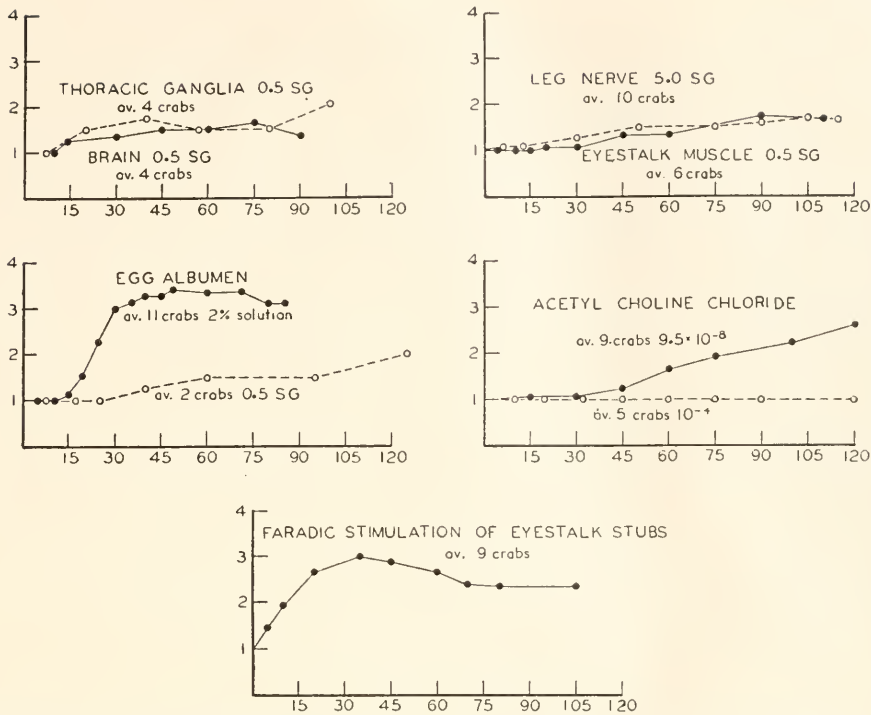


FIGURE 2. Responses of *Hemigrapsus melanophores*. Explanation same as for Figure 1.

carapace width. This made it possible simply to measure the carapace width and to inject the proper amount. Injections were made at the base of a walking leg.

The strength of each extract is expressed in terms of the volume of a sinus gland of the crab injected. Thus "0.5 SG" (cf. Fig. 1) indicates that a crab injected with 5 per cent of its body weight of this extract received a volume of tissue approximately equal to 0.5 times the volume of one of its own sinus glands.

EXPERIMENTS AND RESULTS

The results of the injections are shown in Table II and in the graphs of Figures 1 and 2. Responses are classified as "weak" when the melanin was not dispersed beyond stage 2, and "good" when stage 3 was reached. The average responses do not include those animals which failed to respond. The validity of averaging the arbitrary figures of the melanophore index is subject to criticism (Parker, 1948, pp. 14-15), and the variability of response, shown in Table II, must be considered when evaluating a response.

By far the most potent extracts were those of sinus glands. Other extracts from comparable volumes of tissue, while in some cases acting as rapidly as sinus gland extracts, did not produce the maximum and sustained responses which always followed injections of the latter. Moreover, the only extracts which always produced 100 per cent "good" responses were those of sinus glands. The more rapid

TABLE II

Summary of experiments

Organ extracted	Strength of extract (vol. of one of own sinus glands = 1)	No. of crabs injected	Responses		
			None	Weak	Good
Sinus gland	0.5 SG	28	0	0	28
Sinus gland	3.0 SG	6	0	0	6
Sinus gland	5.0 SG	3	0	0	3
Medulla terminalis	0.5 SG	11	2	4	5
Medulla terminalis	5.0 SG	15	1	5	9
Medulla interna	0.5 SG	13	4	5	4
Medulla interna	5.0 SG	8	0	1	7
Medulla externa	0.5 SG	8	0	5	3
Lamina ganglionaris	0.5 SG	17	10	5	2
Brain	0.5 SG	8	4	4	0
Thoracic ganglia	0.5 SG	12	8	3	1
Eyestalk muscle	0.5 SG	13	7	5	1
Leg nerve	5.0 SG	21	11	9	1
Egg albumin	0.5 SG	14	12	2	0
Egg albumin	2% sol'n	11	0	2	9
Acetylcholine	9.5×10^{-8}	26	17	5	4
Acetylcholine	10^{-4}	5	5	0	0
Faradic stimulation of eyestalk stubs		10	1	1	8

responses to the weaker (0.5 SG) extract are difficult to understand, but because of the small number of crabs injected with the stronger sinus gland extracts and the extent of individual variability in responsiveness, it is not possible to compare the responses adequately.

The responses to optic ganglia extracts with a concentration of 0.5 SG were in most cases weak, but these extracts probably did contain specific chromatophorotropins, since the responses to 0.5 SG egg albumin solutions were so slight, and the responses to eyestalk muscle extracts of the same concentration were also insignificant. However, the amounts of hormone in these optic ganglia extracts, especially those of lamina ganglionaris, appear to have been close to the threshold for the melanophores of *Hemigrapsus oregonensis*.

The responses to optic ganglia extracts of the concentration 5.0 SG were more definite, although they were much weaker than the responses to sinus gland extracts. It can be safely said, therefore, that the medulla terminalis and medulla interna (and probably the other optic ganglia) contain material that causes dispersion of the melanophores in *Hemigrapsus oregonensis*. The importance of this to the normal crab might be determined by removing the sinus glands without destroying the optic ganglia.

The possibility remained that the melanophores were responding to the acetylcholine present in the extracts. Welsh (1939) found large amounts of acetylcholine in leg nerves and ventral ganglia of *Carcinides* (= *Carcinus*), there being about five times as much in ganglia (about 10 γ /g.) as in fibers (about 2 γ /g.), while Smith (1939) found up to 20 γ /g. in nerve fibers and up to 66 γ /g. in

the ganglia of *Cambarus limosus*. It is improbable, however, that the chromatophorotropic effects of Hemigrapsus ganglia extracts are due to their acetylcholine content, since cholinesterase is probably also present in these extracts (Marnay and Nachmansohn, 1937). Moreover, Abramowitz and Abramowitz (1938) obtained slight responses of the chromatophores in only 20 per cent of the *Uca* they injected with acetylcholine. When injected with 5 per cent of their body weight of 9.5×10^{-8} acetylcholine chloride, each Hemigrapsus received the amount of acetylcholine that would have been present in tissue equal in volume to five times one of its own sinus glands, assuming 50% g. as the concentration of acetylcholine in this tissue. Seventeen out of twenty-six crabs thus injected failed to respond, and five crabs injected with the much stronger 10^{-4} acetylcholine also showed no response. This makes it fairly certain that the responses to optic ganglia extracts were not caused by the acetylcholine contained in them.

Responses to extracts of brain and thoracic ganglia of the concentration 0.5 SG were in most cases weak or absent, and they give little information as to whether or not chromatophorotropins are contained in these organs. A few injections of much stronger extracts of brain resulted in good responses, but these experiments were not well controlled. The good response to about ten seconds' faradic stimulation of one of the eyestalk stubs with a Harvard inductorium shows, however, that substances affecting the melanophores can be released in the eyestalkless Hemigrapsus, most probably from some part of the central nervous system. Deep probing of eyestalkless Hemigrapsus with a hypodermic needle at the base of the third or fourth leg also caused melanin dispersion in some cases. It is possible that the melanin dispersion following injections of muscle extracts and egg albumin solutions is an indirect response, caused by the release of chromatophorotropins from central nervous sources.

It is interesting to note that responses to leg nerve extracts were weak or absent, indicating that if a chromatophore hormone is present in nervous tissue it may be produced by or concentrated in the central nervous system rather than the peripheral nerves.

DISCUSSION

It must be emphasized that these experiments do not compare the total amount of chromatophorotropic hormone available to the animal from one organ with that available from another organ, but indicate that while this hormone is most concentrated in the sinus gland it is not absent from certain parts of the central nervous system. It is entirely possible that in *Hemigrapsus oregonensis* as much or more hormone is present in central nervous system sources as in the sinus glands, although the present work does not provide quantitative information concerning this point. Although Brown (1940) found that 80 per cent of the chromatophorotropic material in the eyestalks of several species of shrimps and crabs was referable to the sinus gland, Smith (1948) has recently presented evidence that only about one-third of the retinal pigment activator in the eyestalks of *Hemigrapsus oregonensis* and two other species of grapsoid crabs resides in the sinus glands. It seems not unlikely that the distribution of the melanophore activator in the eyestalks of *Hemigrapsus oregonensis* is comparable.

The concentration of chromatophorotropins in the histologically specialized and

well innervated sinus gland may represent an adaptation for the storage and more especially the release of active substances in effective amounts and within short periods of time. Production of the active principles themselves might be by nervous tissues in general, or, as seems more likely, might be limited to more or less restricted regions of specialized cells within the central nervous system, including the sinus gland itself. Thus we could imagine that the chromatophorotropins in any given mass of nervous tissue are derived from a relatively few cells, each as specialized as sinus gland cells. These cells could be evenly distributed, resulting in a uniform distribution of hormone throughout the central nervous system, as Brown and Saigh (1946) found for CDH in the isopod, *Idothea baltica*. On the other hand, as in the case of CDH and CBLH in Crago, they could be restricted to a single organ (the tritocerebral commissure, Brown, 1946). The sinus gland, as Turner (1948, p. 561) points out, probably represents the highest evolutionary stage in the differentiation of endocrine tissue from the central nervous system in the Crustacea, and would therefore be expected to contain the highest concentrations of active materials. The experiments reported herein show this to be the case for chromatophorotropins in the sinus gland of *Hemigrapsus oregonensis*.

SUMMARY

1. The melanophores of *Hemigrapsus oregonensis* become punctate after eyestalk removal.

2. Chromatophorotropins, which cause dispersion of the melanin when injected, are present in greatest concentration in the sinus gland, and are also present in the optic ganglia and possibly in the brain and thoracic mass of ganglia.

3. The melanin dispersion in response to electrical stimulation of the eyestalk stubs and to deep probing with a hypodermic needle indicates that some source of releasable chromatophorotropins exists other than the eyestalks.

4. The total amount of chromatophorotropins in the sinus gland is not necessarily greater than in any of the central nervous organs. The specialized structure and the innervation of the sinus gland suggests that its importance lies in its ability to store and rapidly release effective amounts of chromatophorotropins.

5. Injection of sufficient amounts of certain substances, including muscle and gill extracts and egg albumin solution, also induces melanin dispersion in eyestalkless *Hemigrapsus oregonensis*. It is suggested that these substances do not contain chromatophorotropic hormones, but the response to them is the result of a more general stimulation causing the release of chromatophorotropins from central nervous sources.

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SOME EFFECTS OF CENTRIFUGING UPON PROTOPLASMIC STREAMING IN ELODEA¹

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As pointed out by Ewart (1903), protoplasmic streaming was probably observed before the existence of protoplasm as such was recognized. Notwithstanding the many studies that have been made upon this interesting and complicated biological phenomenon, certain of the basic problems such as motive force, function and mechanism of flow remain largely unsolved. This is not surprising, because to understand protoplasmic streaming requires a rather complete knowledge not only of protoplasm itself, but also of the physics and chemistry of streaming as well. In fact, certain theories of protoplasmic structure are inadequate because they fail to account for a suitable structural mechanism to allow for protoplasmic streaming.

Of the extensive literature dealing with protoplasmic streaming, few papers have been published which are concerned directly with the effects of centrifugal force on this process as such, although numerous studies have been made upon protoplasmic viscosity by aid of the centrifuge. Accordingly, it seems desirable to record here the results of some studies made upon protoplasmic streaming in the leaf cells of *Elodea* by use of the ultracentrifuge.

MATERIAL AND METHODS

Leaves of *Elodea canadensis* were removed from a region one to two inches back of the tip of an actively growing stem. They were placed in a dish where about one-fourth inch of the tip was cut off in order that the piece be of suitable size to fit in the cell of the air-driven rotor. The tips were then placed directly in a water mount where they were observed under the high dry and oil immersion lenses. Here observations were made upon the frequency and direction of streaming in the cells of a selected area near the tip of the leaf. The piece was then removed to the rotor of the ultracentrifuge with the long axis parallel to the centrifugal force. It was then centrifuged at forces varying from 135,000 to 350,000 times gravity for intervals varying from five minutes to four hours. After centrifuging, the piece was again removed to a slide and the region formerly studied selected for observation.

Some of the pieces, after having the cells stratified in one direction, were reversed in the centrifuge so that stratification in the opposite direction occurred. Certain of the pieces were killed immediately upon removal from the ultracentrifuge by immersion for a short interval in boiling water.

The minimum time required to stop the centrifuge, remove the piece to a slide and find the area formerly studied was about three to four minutes.

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OBSERVATIONS

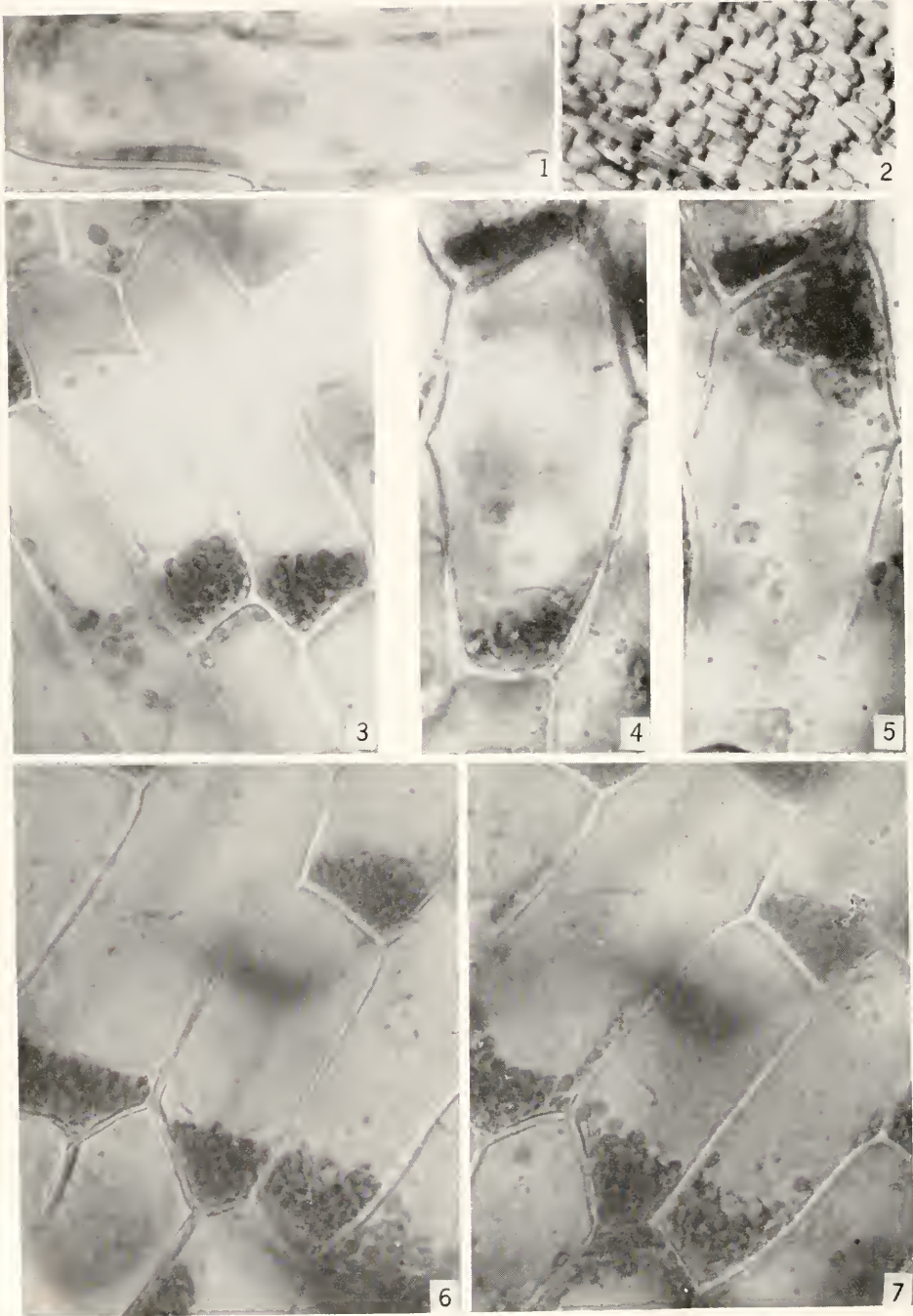
As is well known, the long axis of the cells of *Elodea* is arranged parallel to the long axis of the leaf. Near the mid-rib of the leaf the cells are usually longer and narrower and are more likely to be found undergoing streaming. Usually, if the leaf has been taken from near the tip of an actively growing stem, a few minutes' stand on the stage of the microscope, exposed to light and a slightly elevated temperature, is sufficient to initiate active rotational streaming in a high percentage of the cells. The streaming in *Elodea* has been referred to as rotational (Seifriz, 1943) because the protoplasm is chiefly confined to a peripheral layer between the sides of the cell and the central vacuole. In Plate I, Figure 1, is illustrated a control cell in active streaming. The chloroplasts at the periphery are blurred because of their movement within the interval required to take the photograph.

Under normal conditions, according to Pfeffer (1906), the flow is in one direction only within the cells, clockwise or counter clockwise. In addition, both Pfeffer (1906) and Ewart (1903) maintain that the flow is in opposite directions on the two sides of the dividing walls between each pair of contiguous cells. We have not been able to confirm this statement in the *Elodea* studied here. Counts of a hundred pair of actively streaming adjacent cells revealed that in only about 70 per cent of them was the direction of flow opposite on the two sides of adjacent cell walls. The reason for this discrepancy is not clear, but it should be recalled that Berthold (1866) reported inconsistency in the direction of streaming between adjacent cells of *Elodea*. However, why, in the majority of adjacent cells, the direction of streaming on the two sides of the adjacent walls should be opposite, is unknown.

Figure 2 shows a low power view of a number of cells that were centrifuged at approximately 135,000 times gravity for five minutes. It will be observed that the chloroplasts are packed at the centrifugal pole. If the rotor is allowed to accelerate rapidly, the chloroplasts, but not the cytoplasm, are probably displaced within ten seconds. In other words, the movement of the chloroplasts takes place very rapidly through the protoplasm of the cell. It is of interest that the protoplasm is not killed by this rapid displacement of the chloroplasts through it. In fact, stratified cells (Fig. 4) may be reversed in the centrifuge and the chloroplasts thrown to the opposite end (Fig. 5). This process may be repeated several times without killing the cell. However, Northen and Northen (1938) have found that repeated centrifugation and displacement of the chloroplasts through the cells of *Spirogyra* produces a marked lowering of the viscosity.

High centrifugal force, 350,000 times gravity (Fig. 3), causes rapid displacement of the cellular materials. From the centrifugal to the centripetal pole the elements are stratified as follows: (1) chloroplasts, cytoplasm and nucleus; (2) vacuole. It will be noted that a sharp boundary exists between the centrifugal end of the vacuole and the cytoplasm, although such is not always the case for the chloroplasts, cytoplasm and nucleus. Apparently the vacuole is displaced centripetally and is forced in contact with the cell wall. No cytoplasm can be observed remaining along the sides of the cell. What happens to the plasma membrane if such be a permanent structure in *Elodea*, is not known. No evidence of

PLATE I



organized streaming of the protoplasm is apparent in such cells, although Brownian movement may be evident.

Figures 6, 7, 8 and 9 are a series of photographs showing stratification and partial recovery of three cells centrifuged for thirty minutes at 350,000 times gravity. A complete stratification of the cellular contents occurs here (Fig. 6) as was also noted in Figure 3. Immediate examination upon removal from the ultracentrifuge revealed the cytoplasm in Brownian movement. All indications of any type of organized streaming or even churning movements are absent. However, within thirty minutes after centrifuging, the Brownian movement of the cytoplasm pushes the chloroplasts back beyond the edge of the vacuole so that it is no longer obvious (Fig. 7). A gradual migration of the cytoplasm toward the centripetal end, followed by a movement of some of the chloroplasts along both sides of the cells, occurs. There is in these cells no organized streaming of cytoplasm at this time. Within sixty minutes after centrifuging (Fig. 8), the cytoplasm has become continuous around the sides of the cells and has started to flow in an organized fashion. However, the rate of flow is very slow when compared to that of the normal cells. Ninety minutes after centrifuging (Fig. 9), the chloroplasts in the upper two cells are still largely bunched at the centrifugal end although some isolated chloroplasts are observed being carried around the cell in the stream. In the lower right hand cell the bunched chloroplasts are rotating around the cell as a mass. However, the movement is not continuous but by jerks, due to the forces encountered in trying to move a large mass through a relatively small stream. In some cells treated in this manner, complete recovery with normal streaming and normal distribution of chloroplasts occurs. In others, the cells are killed. In still others, as in Figures 6, 7, 8 and 9, injury occurs from which the cells partially, but not completely, recover within a six-hour interval. Less than one per cent of the cells have their walls ruptured and their contents missing (Fig. 15).

A cell centrifuged at 135,000 times gravity for five minutes is illustrated in Figures 10, 11, 12 and 13. Here the chloroplasts are not as tightly packed as they are in the cells centrifuged at higher forces. Figure 10 shows the cell about four minutes after removal from the centrifuge. In addition to the chloroplasts the nucleus may be clearly observed. Rapid Brownian and churning movements of the protoplasm are evident at the centrifugal end of the cell, and the massed chloro-

PLATE I

All figures in Plates I and II (except Fig. 15) are of living unstained cells of *Elodea*. In all centrifuged cells the force was directed at various angles toward the bottom of the plate except when otherwise indicated.

FIGURE 1. Control cell actively streaming.

FIGURE 2. Low power view of the general effects of centrifugal force on the position of the chloroplasts. Centrifuged at approximately 135,000 times gravity for five minutes.

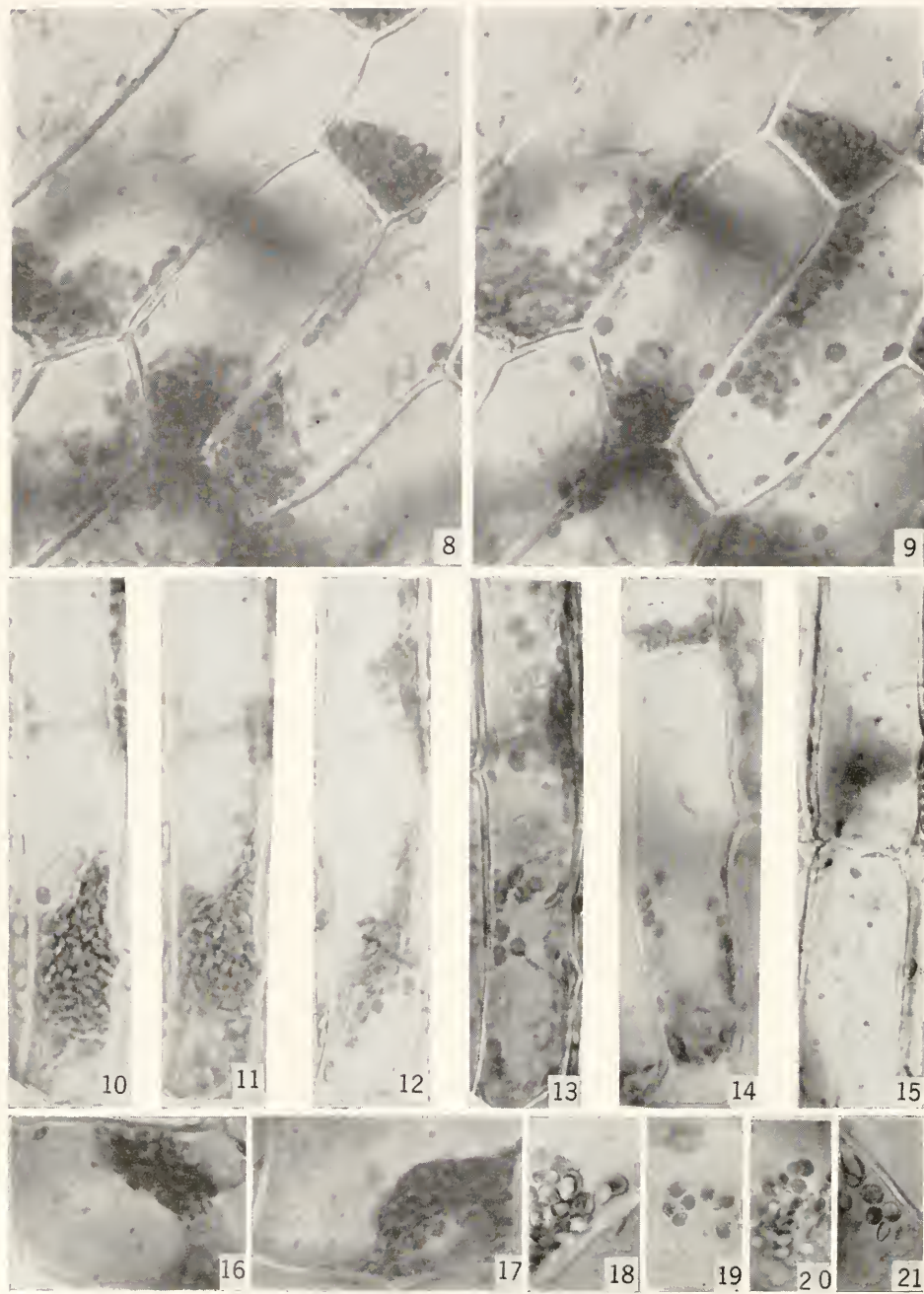
FIGURE 3. High power view of cells showing stratification effects. Centrifuged at 350,000 times gravity for thirty minutes.

FIGURES 4 AND 5. Same cell centrifuged first in one direction and then in the opposite direction (at 135,000 times gravity for five minutes in each case). Centrifugal force in Figure 5 is toward the top of the plate.

FIGURE 6. Group of cells photographed about six minutes after being centrifuged at 350,000 times gravity for thirty minutes.

FIGURE 7. Same cells thirty minutes later.

PLATE II



plasts are being pushed centripetally. Here the reestablishment of flow is up the right side of the cell. The chloroplasts gradually migrate in the stream along the right side of the cell (Figs. 11, 12) and eventually a complete separation of them occurs and recovery is effected (Fig. 13). In this cell the nucleus was not observed to be actively carried about in the streaming cytoplasm. However, they often do rotate in cells so treated. Figure 14 is a cell treated as in Figure 10. Here the redistribution of the chloroplasts is along both sides of the cell instead of one side only. In other words, organized streaming was delayed in this cell for a longer time than it was in the cell in Figure 10, and recovery was eventually established.

In addition to the methods of recovery already described for centrifuged cells, other conditions may occur. In some cells the packed chloroplasts at the centrifugal end may be forced back as a mass to a position near the middle or even to the centripetal end (Figs. 16, 17). This seems to occur as a result of the clumped chloroplasts partially blocking the movements of the redistributing cytoplasm. Consequently, they are simply forced ahead of it. The nucleus, too, may be carried along with the massed chloroplasts. When the cytoplasm breaks around the chloroplasts or develops new streams across the cell, the clumped chloroplasts break away into the stream, usually in small groups at first, but eventually they become isolated. It may take thirty minutes or more for normal distribution of the chloroplasts to become established.

Observations on the direction of streaming in one hundred cells that were subsequently centrifuged at 135,000 times gravity for five minutes, showed that approximately 7 per cent of them had undergone a reversal in direction of flow.

Clumped chloroplasts such as those shown in Figures 16 and 17 sometimes, but not always, cause formation of protoplasmic strands which cross between the wall and vacuole in almost any direction, resulting in many diverse and irregular patterns of flow. Two or more protoplasmic strands may join on the upper surface of the vacuole, giving rise to a churning motion of the protoplasm at the point of juncture. Flow in a single strand may be in opposite directions. When this condition occurs, the velocity of flow is much slower at the point of contact of the two opposite flowing protoplasmic streams. The small granules moving in

PLATE II

FIGURE 8. Same cells sixty minutes later.

FIGURE 9. Same cells ninety minutes later.

FIGURES 10, 11, 12 AND 13. Series of photographs showing recovery from centrifuging at 135,000 times gravity for five minutes.

FIGURE 14. Cell in process of recovery from centrifuging at 135,000 times gravity for five minutes.

FIGURE 15. Cell showing broken wall and chloroplasts missing. Centrifuged at 350,000 times gravity for thirty minutes. Such conditions are rare.

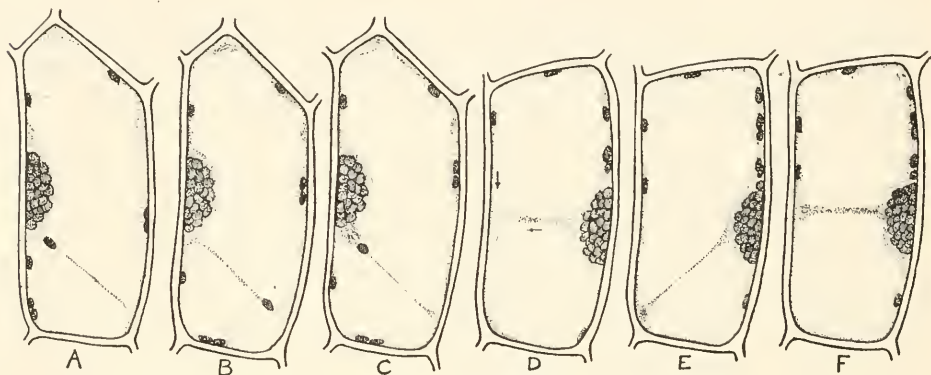
FIGURES 16 AND 17. Cells centrifuged at 135,000 times gravity for five minutes. Photograph taken ten minutes later. The chloroplasts are being forced back from the centrifugal end by Brownian movement of the cytoplasm. (Centrifugal force directed to left in figures.)

FIGURES 18, 19 AND 20. Starch grains in various stages of displacement within chloroplasts. The starch grains are on end of chloroplasts originally directed centrifugally. Brownian movement has caused the chloroplasts to lose the position taken up in the centrifugal field.

FIGURE 21. Starch grain that has been freed from the chloroplast presumably by the centrifugal force.

opposite directions at the point of contact of the two streams may be observed to collide and bounce around each other. Streaming in opposite directions within a single strand is usually a temporary condition. Direction of flow in the strands has also been observed to be in one direction for a time and subsequently reversed. Reversal of flow, however, is usually of short duration and appears to be caused by the difference in flow pressure produced by the main channels of the cytoplasm along the two opposite sides of the cells in which the strands are usually directly or indirectly connected. The difference in flow pressure of the two opposite sides of the cells is often due to the massed chloroplasts blocking the normal channels of flow. Hence, strands are developed at the points of least resistance to the protoplasmic flow.

In some of the thin protoplasmic strands which extend across the cell, a chloroplast may become included within its streaming cytoplasm (Text Fig. 1, A). The chloroplast, being greater in diameter than the stream, moves relatively slowly along its channel (Text Fig. 1, B). In a few such cases we have observed that



TEXT FIGURE 1

FIGS. A, B AND C. Centrifuged cell partially recovered showing movement of chloroplast by means of contraction within small strand. At A the chloroplast is flowing slowly toward the lower right side of cell. At B it has almost reached the opposite end of strand. However, suddenly a rapid backward movement of the chloroplast occurs as shown at C.

FIGS. D, E AND F. These figures illustrate contraction of a cytoplasmic strand. Figure D shows position of strands. At E strand has moved with current down along side of cell. Figure F shows return of strand to near former position.

a quick return of the chloroplast from a position indicated in Text Figure 1, B to that of Text Figure 1, C occurs. Here the reversed movement of the chloroplast seems to be due to a contraction of the strand, because its movement is much too fast to be accounted for as a simple reversal of flow of the cytoplasm within the strand.

Other protoplasmic strands extending across the cell from the upward channel of flow of one side of the cell to the downward channel of flow of the other side sometimes change position rapidly (Text Fig. 1, D, E and F). For example, the end of the strand in connection with the downward channel of flow may move with the current to a position indicated in Text Figure 1, E. When this occurs

the strand appears to be stretched and its diameter diminished. However, suddenly such a strand may "snap back" very quickly to a point near its former position (Text Fig. 1, F). This change seems to be due to a property of the protoplasm of the strand as a whole. The contraction described above may be repeated several times. We have also observed that "balls" of protoplasm considerably larger than the diameter of the strand may flow along its channel. Such occurrences are not rare and have been described by others in normal protoplasmic streaming.

Figures 18, 19, 20 and 21 are centrifuged cells with chloroplasts showing starch grains. The starch inclusions appear heavier than the chloroplasts and are displaced centrifugally. Figure 18 illustrates a chloroplast with the starch grain displaced so that it appears to be partially extruded. The positions taken up by smaller starch grains are indicated in Figure 19. The upper chloroplast in Figure 20 shows not only a partially displaced starch inclusion, but evidence of a partial stratification of the other materials as well. However, this is rare, for in the majority of the cells little, if any, stratification can be detected within the unstained chloroplast. This indicates either that the chloroplast as a whole is very dense, or that its contents vary only slightly in relative specific gravity.

In Figure 21 the lower inclusion is an isolated starch grain which has probably been pulled away from the chloroplast by the centrifugal force. It is difficult to say anything definite concerning the nature of the surface "membrane" of the chloroplast other than that considerable resistance is met at the surface in the displacement of the starch grains through it. In other words, the presence of some form of limiting "membrane" is indicated, although such a structure could not be seen in the centrifuged unstained chloroplast.

DISCUSSION

It is unnecessary here to review the extensive literature dealing with protoplasmic streaming, since excellent reviews on this subject have been published by Ewart (1903) and more recently by Seifriz (1943).

Protoplasmic streaming is known to be affected by many agents such as temperature, visible light, ultraviolet light, salts, acids, alkalies, oxygen, organic substances, anesthetic agents, x-rays, radium, electricity, hydrostatic pressure, mechanical manipulation and supersonic waves (see Seifriz, 1943, for references). In this paper it has been demonstrated that it is also affected to varying degrees by ultracentrifugal force. However, both Andrews (1915) and Vexler (1935) report that centrifuging with the usual laboratory type centrifuge produces little effect upon protoplasmic streaming. In fact Vexler (1935) found that centrifuging stimulated streaming in *Myxomycetes*.

Marsland (1939) has suggested that in *Elodea* the streaming is motivated by sol-gel reactions and consequently is a phenomenon fundamentally related to amoeboid movement. The evidence for this is that with increasing hydrostatic pressure, the rate of protoplasmic streaming in *Elodea* is diminished. Complete obliteration of streaming with decreasing viscosity of the cytoplasm occurs at between 400 to 500 atmospheres. This reaction is reversible. To apply the theory of sol-gel reversibility of protoplasm as the chief mechanism of streaming in a cell like *Elodea* is not easy, for according to Pfeffer (1906) only a very thin ectoplasmic membrane

exists in contact with the cell wall in actively streaming cells. Furthermore, according to Ewart (1903), the adjacent surfaces of both the protoplasm and vacuole flow. In addition, to account for rotational streaming by a sol-gel mechanism requires several assumptions. Because of this, Seifriz (1943) suggests an alternate view, namely, that "streaming is occasioned by a contractile force which need not involve a viscosity change or a sol-gel transformation."

If the sol-gel mechanism is the main source of the motive force in protoplasmic streaming in *Elodea*, as there is certainly good evidence that it is for protoplasmic streaming in certain other cells (Mast, 1931; Lewis, 1942), the inhibition of streaming produced by centrifuging may be due to a lowering of the viscosity resulting in a disruption of the sol-gel mechanism.

Moore (1935) found that centrifuging at 75,000 times gravity for five minutes deforms and retards proliferation (and I presume streaming) for fifteen hours in plasmodium. He interprets this result as due to a separation of a heavy and light component of the cytoplasm. When allowed to stand for sufficient time, these heavy and light components of the cytoplasm return to their normal spatial relationships, and proliferation and streaming are reestablished.

High centrifugal force has been demonstrated to displace most of the visible cellular materials (Beams and King, 1939; Beams, 1943) and certain ultramicroscopic structures in the liver cell (Claude, 1943), as well as various types of protein molecules in non-living colloidal solutions (Svedberg, 1934). Yet, convincing evidence has not been obtained that a disruption of the vital ultramicroscopic organization of the protoplasm of *Ascaris* eggs occurs even at forces on the order of 900,000 times gravity for thirty minutes (Beams and King, 1937; Beams, 1943). This indicates that the intermolecular forces contributing to the vital structural protoplasmic framework are sufficiently great to resist disruption by high centrifugal force.

However, whether or not the inhibition of protoplasmic streaming in *Elodea* reported here may be explained on the same basis as that given by Moore (1935) for plasmodium is unknown.

In spite of the fact that all the visible cellular materials, including the cytoplasm, may be displaced to one end of the cell, recovery in the majority of cells does not result in a reversal of the direction of streaming. No experimental procedure has as yet been developed to produce consistent reversal in streaming of *Elodea* cells. However, Ewart (1903) states that change in direction of streaming can sometimes be observed in cells of *Elodea* after the application of stimuli sufficiently great to produce death in some cells and temporary stoppage of streaming in others. Seifriz (1943) has observed in treated *Elodea* that the chloroplasts flow as a belt around the "waist" of the cell. It is difficult to understand where the factors for polarity of streaming reside within the cell. All of the cytoplasm, including the peripheral membrane, seems to be displaced, at least insofar as could be detected with the microscope.

Both elasticity and contractility have been observed in streaming cytoplasmic strands. In thin strands the thickness of the cortical layers must be very small. Hence, the contractile properties of the strand probably include the whole of its protoplasmic structure.

The chloroplasts seem to be of a highly viscid consistency. The starch granules were the only elements within them that could be consistently displaced by high

centrifugal force. This is in agreement with the observations made with the micro-dissection apparatus, to the effect that chloroplasts appear to be composed of an "elastic jelly of doughy consistency" (Scarth, 1927). Large starch grains often appear in normal storage tissue attached to the surface of a chloroplast (Zirkle, 1926). However, there seems little doubt that the displacement of the starch grains reported here is due to centrifugal force.

CONCLUSIONS

High centrifugal force produces a rapid stratification of the visible cellular materials in *Elodea*. From the centrifugal to the centripetal end, the materials are stratified as follows: (1) chloroplasts, cytoplasm and nucleus; (2) vacuole. In cells examined immediately after centrifuging at high forces, evidence of organized streaming is usually absent, but Brownian movement is often apparent. Most of the cells survive exposure to 350,000 times gravity for thirty minutes.

The inhibition of streaming may be due to a lowering of the viscosity as a result of the rapid displacement of the chloroplasts through it.

Recovery from centrifuging usually occurs in the following order: (1) Brownian movement at the centrifugal end aids in a redistribution of the granular cytoplasm along one or both sides of the cells; (2) initial streaming movements are usually of an unorganized type; and (3) organized streaming is slowly established which results in a slow irregular rotation of the large massed chloroplasts. Eventually the bunched chloroplasts separate, and the normal velocity of streaming, as well as the normal distribution of chloroplasts, is established.

In only about 7 per cent of the cells could the direction of streaming be reversed by centrifuging.

Evidence of elasticity and contractility within thin strands of protoplasm has been observed.

Displacement of starch grains within chloroplasts has been observed.

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THE PRESENCE OF THE TRICARBOXYLIC ACID CYCLE IN THE CILIATE COLPIDIUM CAMPYLUM¹

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Colpidium campylum is a ciliate which can be easily cultured bacteria-free in a liquid medium. It has been demonstrated that when cultured in proteose-peptone from which the lipids have been extracted, the organism is capable of synthesizing large amounts of fatty acids (Wilber and Seaman, 1948).

Since the tricarboxylic acid cycle is a link between protein and carbohydrate metabolism, it seemed desirable to make a study of this cycle as the first step toward the elucidation of the pathway for fatty acid synthesis from protein in this organism.

While there have been many investigations of this cycle in vertebrate tissue and in bacteria, there has been little done with protozoa. Van Niel, Thomas, Ruben and Kamen (1942) found that the ciliate *Tetrahymena geleii* assimilates carbon dioxide in the anaerobic formation of succinate during the fermentation of glucose. Baker and Baumburger (1941) found cytochrome c, b, and a₁ to be present in this same organism with indications of the presence of cytochrome a₂. Hutchens, Jandorf and Hastings (1941) ascertained the DPN content of the flagellate *Chilomonas paramecium*. Hutchens (1940) also identified the presence of cytochrome c in *Chilomonas*. Laurie (1935) demonstrated the presence of succinic dehydrogenase in the ciliate *Glaucoma pyriformis*.

MATERIALS AND METHODS

Colpidia were grown in sterile, pure cultures in 150 cc. Erlenmeyer flasks containing 50 cc. of 3 per cent Difco proteose-peptone solution from which the carbohydrate had been precipitated with copper sulfate (Peters and Van Slyke, 1931) and the lipids extracted with hot alcohol (Bloor, 1943). The organisms used were obtained from cultures maintained in the Biological Laboratory, Fordham University and are the same strain as was used in a previous investigation (Wilber and Seaman, 1948). For use in this investigation, new cultures were inoculated with 1 cc. of organisms from a three-day culture and allowed to grow for two days at a temperature of $22 \pm 2^\circ$ C. At this time the cultures were at the mid-point of the logarithmic phase of growth (population about 40,000 colpidia per cc.).

The organisms for use were concentrated by centrifugation and aliquots with-

¹ Portion of a dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Fordham University.

² The author wishes to express his sincere gratitude to Dr. Charles G. Wilber for invaluable aid and encouragement.

³ U. S. Public Health Service Fellow.

drawn for ascertaining the dry weights of the cells (Ormsbee, 1942). The remaining cells were washed three times with Hahnert's solution (Hahnert, 1932), to which was added magnesium sulfate to make a final concentration of 0.02 M (final pH adjusted to 5.6). The cells were then starved for twelve hours before use. At the end of this period the organisms were again concentrated, resuspended in the modified Hahnert's solution and 2 cc. portions (containing approximately 10 mg. dry weight of cells) transferred into standard Warburg vessels.

Oxygen uptake was measured by the conventional Warburg direct method. In all cases the total volume of each vessel was 3.5 cc. Vessels were shaken at a rate of 120 cycles per minute through an arc of 5 cm.

Sodium pyruvate was prepared by the method of Robertson (1942); oxaloacetic acid by the method of Krampitz and Werkman (1941). All other substrates were obtained commercially. Concentrations of substrates are given as final concentration.

Pyruvic and α -ketoglutaric acids were estimated according to the method of Friedmann and Haugen (1943); succinic acid according to Krebs (1937); oxaloacetate according to Edson (1935); fumaric acid according to Krebs, Smyth and Evans (1940).

RESULTS

Pyruvate is rapidly metabolized by *Colpidium*. When 0.02 M pyruvate is added to cells respiring in modified Hahnert's solution there is an immediate

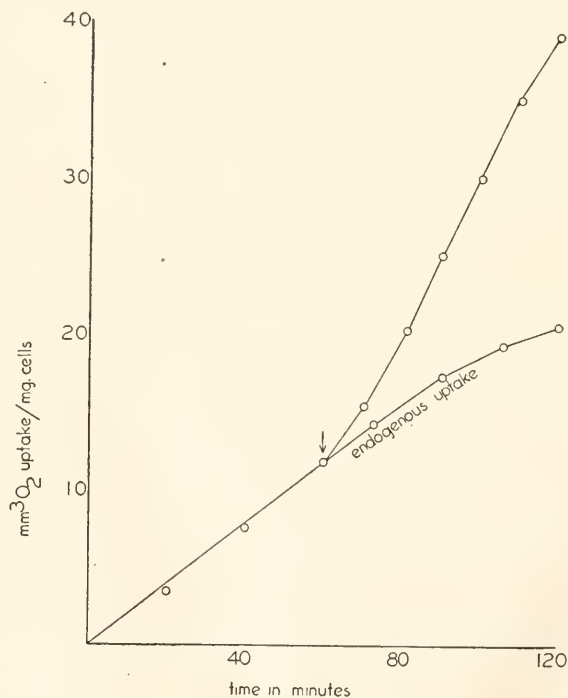


FIGURE 1. Effect of pyruvate on oxygen uptake in *Colpidium*. Modified Hahnert's solution, pH 5.6. Gas phase, O₂. Temperature, 25.5° C. At arrow, 0.02 M pyruvate added.

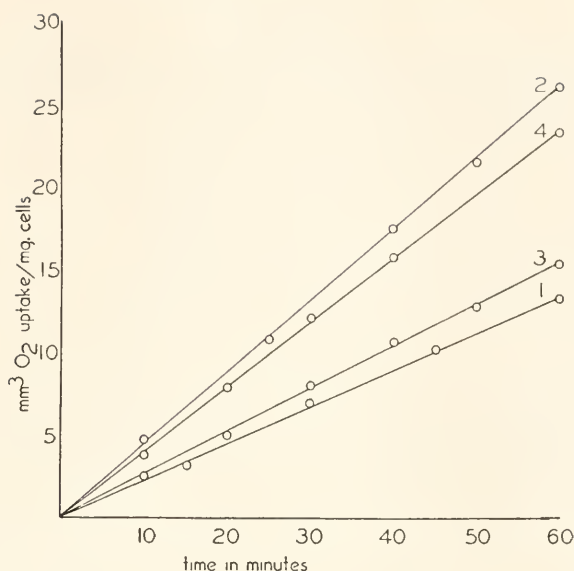


FIGURE 2. Effect of malonate and fumarate on oxygen uptake in *Colpidium*. Modified Hahnert's solution, pH 5.6. Gas phase, O_2 . Temperature, $25.5^\circ C$. Curve 1, no added substrate; curve 2, 0.02 M pyruvate; curve 3, 0.02 M pyruvate + 0.02 M malonate; curve 4, 0.02 M pyruvate + 0.02 M malonate + 0.001 M fumarate.

increase in the rate of oxygen uptake (Fig. 1). There is a utilization of 0.081 mg. of pyruvate per mg. dry weight of cells per hour (Table II).

If the tricarboxylic acid cycle plays a role in the metabolism of *Colpidium*, the oxygen uptake in the presence of pyruvate should be inhibited by malonate. This inhibition should be released upon the addition of fumarate. Figure 2 shows that 0.02 M pyruvate increases the Q_{O_2} from the endogenous value of 13.2 to 26.3, an increase of 99 per cent. In the presence of pyruvate and 0.02 M malonate the Q_{O_2} is 15.4, 83 per cent inhibition of the pyruvate effect. The Q_{O_2} is restored to a value of 23.6 by the addition of 0.001 M fumarate, an 89 per cent recovery of the malonate inhibition.

The effect of other acids of the tricarboxylic acid cycle on oxygen uptake is shown in Table I. Succinate results in an increased Q_{O_2} of 105 per cent; α -ketoglutarate 102 per cent; fumarate 90 per cent; malate 97 per cent; and oxaloacetate, an increase of 85 per cent.

The quantities of metabolites recovered from various substrates are shown in Table II and III. Fumarate and α -ketoglutarate are recovered in approximately equal amounts when pyruvate is the substrate. The addition of fumarate to pyruvate increases the recovery of α -ketoglutarate by 142 per cent. Fumarate and pyruvate are recovered in a ratio of approximately 1 to 4 when oxaloacetate is utilized as a substrate.

Table III shows that as a result of the fumarate release of malonate inhibition, there is an added utilization of 0.051 mg. pyruvate per mg. dry weight of cells per hour, and an added recovery of succinate amounting to 0.013 mg. per mg. dry weight of cells per hour.

TABLE I

Effect of acids of the tricarboxylic acid cycle on oxygen uptake in Colpidium. Modified Hahnert's solution, pH 5.6. Gas phase, O₂. Temperature, 25.5° C. Concentration of all substrates except fumarate, 0.02 M; fumarate, 0.001 M.

Q _{O₂}	Substrate
13.2	—
27.1	succinate
26.8	α -ketoglutarate
25.1	fumarate
26.1	malate
24.5	oxaloacetate

TABLE II

Utilization of substrates and recovery of intermediate metabolites in Colpidium. Modified Hahnert's solution, pH 5.6. Gas phase, O₂. Temperature, 25.5° C. Q_{substrate} is mg. substrate utilized (—) or mg. metabolite formed (recovered) (+) per mg. dry weight of cells per hour. Pyruvate, 0.02 M; oxaloacetate, 0.002 M; fumarate, 0.001 M.

Q _{substrate}	Substrate added		
	pyruvate	pyruvate + fumarate	oxaloacetate
(+)pyruvate	—	—	0.044
(—)pyruvate	0.081	0.065	—
(+)ketoglutarate	0.012	0.029	—
(—)oxaloacetate	—	—	0.121
(+)fumarate	0.016	—	0.014

TABLE III

Formation of succinate in Colpidium. Modified Hahnert's solution, pH 5.6. Gas phase, O₂. Temperature, 25.5° C. Malonate, 0.02 M; pyruvate, 0.02 M; fumarate, 0.001 M.

Q _{substrate}	Substrate added (in addition to malonate)	
	pyruvate	pyruvate + fumarate
(—)pyruvate	0.016	0.067
(+)succinate	0.003	0.016

TABLE IV

Effect of succinate, α -ketoglutarate, and citrate in releasing malonate inhibition in Colpidium. Modified Hahnert's solution, pH 5.6. Gas phase, O₂. Temperature, 25.5° C. Malonate, pyruvate, α -ketoglutarate, succinate, 0.02 M; citrate, 0.008 M; fumarate, 0.001 M.

Substrate (in addition to pyruvate which was present in all vessels)	Q _{O₂}
— — — —	26.3
malonate	15.4
citrate	26.1
citrate + malonate	13.7
citrate + malonate + fumarate	22.8
α -ketoglutarate + malonate	28.4
succinate + malonate	27.6

Added citrate in final concentrations ranging from 0.002 M to 0.01 M has no effect on the oxygen uptake. Table IV shows the ability of citrate in releasing malonate inhibition as compared to the ability of succinate and α -ketoglutarate to release the inhibition. Succinate and α -ketoglutarate release the malonate inhibition to approximately the same extent as does fumarate (compare Fig. 2), whereas citrate does not release the inhibition.

DISCUSSION

It would be desirable to compare the Q_{O_2} values obtained for Colpidium in this investigation with values obtained for other protozoa. However, it is impossible to make such a comparison, since it was found (Ormsbee, 1942) that the Q_{O_2} of the same species of Tetrahymena varies from 6.2 to 77.7 depending upon the age of the culture, the length of the starvation period before oxygen uptake is measured, and the composition of the suspending medium. Other factors affecting Q_{O_2} values in protozoa are the rate of shaking of the manometer vessels (Hall, 1938) and the concentration of cells used (Pace and Lyman, 1947). Hutchens (1941) found that in *Chilomonas paramecium* the oxygen uptake per hour per 10,000 cells varies with different strains, even though both strains are studied under identical conditions.

Since added citrate does not increase oxygen uptake or release malonate inhibition, and since fumarate, succinate, and α -ketoglutarate do cause increased oxygen uptake and do release malonate inhibition, it must be concluded (Stare, Lipton, and Goldinger, 1941) that citrate does not occupy a major position in the tricarboxylic acid cycle as it occurs in Colpidium.

It appears from the data of Von Dach (1942) that the tricarboxylic acid cycle is not present in the colorless flagellate, Astasia. In this organism, succinate, fumarate and malonate have no significant effect on the oxygen uptake. In *Paramecium caudatum*, succinate increases oxygen uptake by only 8 per cent (Leichsenring, 1925).

Elliott (1935) found that pyruvic acid (0.5%) inhibits growth in *Colpidium campylum* and in *C. striatum*. On the other hand, Bond (1933) found that pyruvic acid stimulated growth in *C. campylum*. However, he found that succinate (1.0%) and malate (1.0%) inhibit growth. These findings are unusual if, as has been demonstrated in this paper, these compounds are metabolites. It must be noted that the concentrations used by these authors were very much higher than those used in the present investigation. It is well known that normally occurring metabolites in high concentrations may cause inhibition of metabolic functions, as measured by oxygen uptake. It would be desirable to ascertain the effects of acids of the tricarboxylic acid cycle on the growth of Colpidium when used in concentrations which are known to be physiologically active (0.001–0.02 M). Such an investigation is now in progress.

SUMMARY

1. Evidence is presented for the presence of the tricarboxylic acid cycle in the metabolisms of the ciliate *Colpidium campylum*.

2. Apparently citrate does not occupy a major position in the tricarboxylic acid cycle as it occurs in Colpidium.

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GAMETOGENESIS IN THE OYSTER UNDER CONDITIONS OF DEPRESSED SALINITY

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The American oyster, *Ostrea virginica* Gmelin, flourishes naturally in brackish waters ranging in salinity from 16 to 27 parts per thousand. But the salt toleration of the animal is such that it can survive in waters having a much broader range of salt content. In many localities, commercial production of oysters is maintained where seasonal floods may expose the bars to entirely fresh water for short periods of time. Some of the more important seed-producing areas on the Atlantic Coast consistently have a salt content of less than 15 ‰. Consequently the effects of lowered salinity on oyster physiology and reproductive ability have long been of interest. The opportunity presented itself in 1946 to examine the gonads of oysters living under unusually great variations in salt content. Extensive flood waters from the Susquehanna River watershed into the upper reaches of Chesapeake Bay during the summer of 1945 and spring of 1946 caused salinity depressions from a normal range of 10 to 15 ‰ to zero for protracted periods. Oyster beds located twenty miles south of the entrance of the river into the bay were frequently exposed to fresh water. In the period following these extremes, mortalities up to 70 per cent of the population were recorded on the bars in this area (Engle, 1946). The oysters remaining viable were of unusually poor quality. The body tissues were edematous and nearly transparent. The adductor muscle lacked tonus so that the valves could be separated easily and frequently were gaping.

Samples of ten or more oysters from this low salinity area, designated here as the LS group, were collected weekly in the summer and at longer intervals during the fall and winter of 1946. Transverse sections of the gonad were prepared for histological examination. For comparative purposes, a similar series of oysters, designated as the HS group, was collected in another part of the bay where the salinity was higher and remained relatively unaffected by the flood conditions. These oysters were of good market quality and during the summer produced a set of young oysters of commercial proportions indicating normal gonad development and spawning reactions. Routine hydrographical observations were made at the time of each sampling, as well as plankton tows and notes on the feeding activity and general condition of the oysters.

Of the 185 oysters in the LS group over three years old examined, 40 per cent were females, 33 per cent were undifferentiated, 26 per cent were males and 1 per cent were sex reversals. Of the 221 specimens in the HS group, 70 per cent were females, 29.5 per cent were males and 0.5 per cent were hermaphroditic. The absence of undifferentiated gonads in the HS group was striking in comparison with the LS group.

The orderly sequence of events in the development of functional gametes in the American oyster has been described (Coe, 1932; Loosanoff, 1942), and the résumé of the stages given here for the HS oysters growing in Chesapeake Bay differs in no important respect from conditions found elsewhere except with regard to timing (Loosanoff and Engle, 1940). Spawning is initiated when water temperatures rise to levels approximating 18 to 20° C., and consequently its occurrence varies from year to year at any particular geographical location. Typically, after the final spawning of the population in late summer, there is a short period of rest in which the gonadal tissue is made up of undifferentiated gonial cells. These soon proliferate and early maturation takes place. By this time, usually late December in Chesapeake Bay, water temperatures have decreased to the extent that the oyster becomes inactive and the gonad remains quiescent until the following March. Thus in early spring, gonad sections from the HS oysters are characterized by fairly large numbers of auxocytes. As water temperatures increase, differentiation and growth proceed at a rapid pace, and mature gametes first appear in May when spawning may begin. In June most of the gonads are filled with ripe sexual products, and from that time until early September, successive waves of spawning may continue. By the end of September the majority of gonads are in the resting condition.

In contrast to this typical picture, section of the gonads of the LS group revealed that 5 to 40 per cent of each sample contained gonads which were in the resting

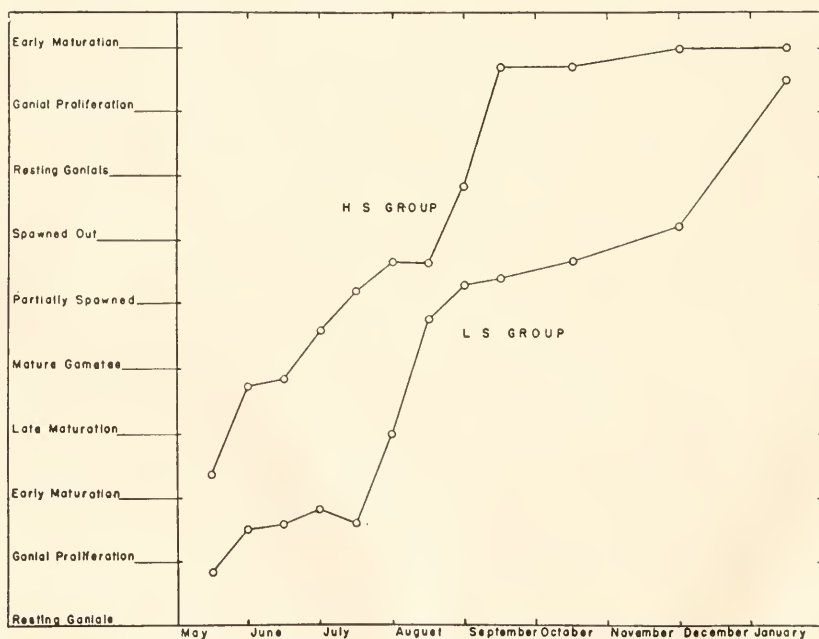


FIGURE 1. Seasonal progression of stages in the development of gametes in oysters from a low salinity area (LS Group) and from a higher salinity area (HS Group). Each point represents the predominant activity in a sample of 10-40 oysters collected during a two-week or longer period. Initial and final phases in the growth of auxocytes, termed Early and Late Maturation, are normally separated by the winter hibernating period in this area.

gonial or undifferentiated stage until the middle of August. This condition must have persisted from the close of the spawning period of the previous year, the time of its normal occurrence. By the end of August there was a marked improvement in the appearance of the oysters, and the gonads reached stages of activity which had characterized the HS oysters examined two months earlier. In early November, a majority of the LS oysters were spawned out, and from this time until January, early maturation continued at a high level. When the oysters finally entered the hibernating stage at temperatures of less than 5° C., the majority of LS oysters were indistinguishable, with respect to the histology of the gonad, from the oysters living in the higher salinity area.

In about 90 per cent of the specimens of LS oysters examined, the gametogenic cycle lagged approximately two months behind that of the high salinity group, but in the remaining 10 per cent of the specimens, the gametogenic cycle showed the same timing pattern as in the HS group. In order to portray graphically the differences between the two populations, the successive stages in normal gonad activity were assigned arithmetic values from one to ten, depending on the preponderant condition or cell type present. The average arithmetic value was then obtained for each sample of gonads collected over a two-week or longer period and has been plotted against time for the two areas studied (Fig. 1).

It was observed that during the summer, developmental stages in different gonads of a sample overlapped or were concurrent because of the relatively long period of four months in which eggs are produced. The average values shown in Figure 1 demonstrate the seasonal trend of the gametogenic cycle, but they do not show the wide variations found within each of the samples of oysters. Earlier investigators (Nelson, 1928; Loosanoff, 1942) have noted the variations in gonad response found in some individuals of a sample where, for unknown reasons, maturation may be delayed or physiologically mature gametes may be retained long after the general population has spawned. This condition is especially prominent in the LS oysters examined. In the first week of August, individuals from one sample demonstrated all stages in gonad development from the undifferentiated gonial cells to the spawned-out stage. The degree of variation among individuals was far less extensive in the HS group, in which for the same period the gonads were fairly equally divided between the partially spawned and the spawned-out stages. The percentage distribution of each stage within the samples collected is tabulated to illustrate this disparity in the two populations (Fig. 2).

During the first two weeks of August there was a significant change in the appearance of the gonad sections from the oysters of the LS group. Wide variations in the stages of activity attained by the individual oysters continued, but all of the gonads suddenly advanced beyond the indifferent and early maturation stages, and 50 per cent of them were partially or almost completely spawned. In this period there were only minor fluctuations in the temperature but the salinity rose abruptly from less than 3 ‰ to more than 8 ‰. No other environmental changes of importance were noted during this period.

The recovery of oyster larvae from the plankton tows made at the two stations corresponds, in general, with the histological picture.¹ In the high salinity area,

¹ The writer is indebted to Mr. James B. Engle of the U. S. Fish and Wildlife Service, who provided the data on plankton.

larvae were found two weeks after mature gametes were observed in the sections. The last plankton sample taken, October 8, still contained numerous larvae, although it was from two to three weeks after the apparent absence of gametes from the gonad sections. There were two seasonal peaks in larval production: the first week of July and the last week of August. In the low salinity area there was but one seasonal peak toward the end of August. No larvae at all were collected here until seven weeks after their initial appearance in the HS area, although throughout this period 10 per cent of the gonad sections had contained apparently mature gametes. The failure to find larvae in the water at that time may be attributed to inadequate sampling methods or, more probably, to the inhibition of spawning. The observed tissue edema may have interfered with the activity of the adductor muscle in the spawning reaction (Galtsoff, 1938) or have partially closed the gill ostia, thus preventing the passage of ova to the exterior (Hopkins, 1936).

	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H
EARLY MATURATION													25	9	74	11	75	100
GONIAL PROLIFERATION													5	21	11	20	30	45
RESTING GONIALS											4	9	10	9	5		5	
SPAWNED OUT						10	20	16	63	30	55	9	50	9	11		20	
PARTIALLY SPAWNED					6	37	80	37	20	41	82	10	55		56		40	
MATURE GAMETE		11	7	75	16	84	12	53	24	47	50			18	11			
LATE MATURATION		39	7	25		16												
EARLY MATURATION	32	39	36	4	32		35		21	5								
GONIAL PROLIFERATION	21	11	29		26		41		21	5								
RESTING GONIALS	47		21		26		6		34	27								
	MAY 16-31	JUNE 1-15	JUNE 16-30	JULY 1-15	JULY 16-31	AUGUST 1-15	AUGUST 16-31	SEPTEMBER 1-15	SEPTEMBER 16-30	OCT	NOV.	DEC.	JAN					

FIGURE 2. The percentage distribution of different stages of gonad activity in each sample of oysters collected from the low (L) and high (H) salinity areas. See legend under Figure 1 for description of samples.

Water temperatures throughout the period of observations were normal for the region (Fig. 3). In the low salinity area, the bottom temperature was 16.8° C. in the middle of May, approached 20° C. in the first week of June, and reached the summer maximum of 26° C. on the first day of August. It then decreased gradually to 15.8° C. at the end of October, and to less than 5° C. in the period December through January. Bottom water temperatures in the high salinity area regularly followed the same levels within one or two degrees.



FIGURE 3. Seasonal fluctuations in salt content of bottom waters in the high (HS) and low (LS) salinity areas. The bottom temperature curve (T) is shown for the LS area. Temperatures in the HS area did not vary more than one degree plus or minus from these data.

The salt content of the water was more variable (Fig. 3). In the LS area, bottom salinities fluctuated from zero (fresh) to 6 ‰ in the period from the middle of May until the first of August. One-third of the records for this time showed fresh water. In August the salt level increased steadily to 13 ‰ and then gradually dropped to 11 ‰ by the end of the year. In the HS area, the lowest salinity of 6 ‰ was recorded toward the end of June. Before and after that time, the salinity increased steadily to 15 ‰.

Only four specimens, one per cent of the total examined, gave evidence of the instability of the sex mechanism in this species of oyster. Three of the individuals were clearly defined protandric reversals in which the gonaducts contained residua of spermatozoa, and the walls of the follicles were lined almost exclusively with oocytes in early stages of maturation. These specimens were obtained early in September, which indicates that reversal of sex had taken place when the majority of the population were spawning. Loosanoff (1942) has suggested that sex

reversal takes place when the gonads are made up of undifferentiated gonial cells, usually in late October in Long Island Sound. It would appear most reasonable that sex reversal should take place during this stage, but the specimens found here suggest that if it does, the indifferent stage may occur much earlier in the summer, i.e., July and August, in at least part of the population. In one of the four specimens mentioned above, the gonadal tissue was made up of fairly equal numbers of developing oocytes and spermatocytes within each follicle. The developmental stages attained were the same as in other unisexual specimens collected at the same time, indicating that this oyster would have been a functional hermaphrodite when the general population spawned. The percentage of intersexes found in this rather small sampling agrees with observations by other workers. In the 221 oysters from the HS area there was a ratio of females to males of 2.41. This figure is comparable with observations in Galveston Bay, Texas, but contrasts with the approximately 50-50 sex ratio found along the Atlantic Coast (Hopkins, 1931).

The deleterious effect of the environment on the physiology of the oyster, as evidenced by the delayed production of gametes until such time that the water temperatures made their survival improbable, would appear to be due to the low salinity of the water. That this effect was not a direct inhibition of gametogenesis is indicated by the fact that 10 per cent of the LS group elaborated mature gametes at the usual time in the early summer. The factors directly affected by lowered salinities which may be operating here to prevent gametogenesis include several possibilities. It has been shown (Hopkins, 1936) that during exposure to fresh water the oyster's valves may be closed most of the time and also that even when open, the passage of water through the gills may decrease or stop entirely. Either one or both of these factors would seriously curtail the feeding of the animal. It is also possible that during this time necessary food elements were absent from the plankton, or that tissue edema prevented the normal assimilation of food. In any event the end result appears to have been, fundamentally, a tissue starvation. Hopkins (l.c.) theorized such an end result after studying the feeding mechanism in *O. gigas* in the presence of artificially lowered salinities. It was noted that in the small group of LS oysters which produced gametes at the usual time in late spring, there was a moderate reserve of stored food which gave the tissues a typical opaque appearance. These oysters, as well as the ones having no visible food storage, had empty digestive tracts at the time of examination. This would indicate that the reserve food had been held over from the previous fall rather than that this small group had been able to continue feeding during the period of lowered salinities. The evidence is clear, moreover, that soon after the salinity level rose above 6 ‰ in the first week of August, the animals commenced feeding, there was an obvious improvement in the appearance of the tissues, and gonad activity started to approach the normal picture.

SUMMARY

Histological examination of oyster gonads from an area naturally exposed to prolonged periods of fresh water, when compared to oyster gonads from an adjacent, unexposed area, showed:

1. Gametogenesis was inhibited in 90 per cent of the surviving population until salinity levels rose above 6 parts per thousand.

2. Following the salinity increase, oysters rapidly improved in condition but required from three to four months to attain the same final level of gonad activity as the unaffected group.

3. Marked variation and suppression of gonad activity in the exposed oysters is attributed to variations in food availability, rather than to direct inhibition of sexual activity by less saline water.

4. Sex ratios and extent of intersexuality in the population sampled, as well as details of the gametogenic cycle, agree for the most part with published observations on *Ostrea virginica* in other parts of its geographical range.

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NUCLEAR AND CYTOPLASMIC INTERRELATIONS IN THE FERTILIZATION OF THE ASTERIAS EGG

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The existence of a functional relation between nucleus and cytoplasm is generally accepted, but there are relatively few instances in which the relationship can be demonstrated experimentally. Among ova an extreme case is that exhibited by the maturing ovum. Fol (1877, 1879), in his classic work¹ on the maturation and fertilization of the egg of *Asterias glacialis*, was probably the first to associate the maturation of the ovum with the breakdown of the germinal vesicle. In general, the significance of maturation of the ovum has been too closely limited to the elimination of the polar bodies. More attention should be given to what is probably the basic feature of the phenomenon, namely, changes incurred in the cytoplasm through the admixture of nuclear material from the enlarged germinal vesicle of the ovarian egg (cf. maturation cytoplasmique of Delage, 1901; and R. Chambers, 1921). It has been recently proposed (R. Chambers, 1949) that the cytoplasm of the maturing and mature egg be termed *karyocytoplasm*.

There is also to be considered a relationship between the male and female nuclear elements of the fertilized egg and of both elements with the maturing karyocytoplasm. Many observers have ascribed the movements of the male and female pronuclei to their mutual attraction across the intervening cytoplasm of the egg. An early attempt at testing the existence of such an attraction was made by George Lester Kite, a pioneer in microdissection. In a lecture (unpublished) given during the summer of 1915 at the Marine Biological Laboratory, Woods Hole, Dr. Kite described his efforts at interposing the tip of a microneedle as an obstacle between the male and female pronuclei in the transparent egg of *Lytechinus*, then known as *Toxopneustes*. As he dramatically stated: "The pesky nuclei insisted in slipping around the obstacle and no efforts, short of destroying the egg, could prevent the nuclei from approaching one another and uniting."

More recently, E. L. Chambers (1939) was able to offer an interpretation in

¹ Fol's 1879 paper is extraordinary for the abundance and accuracy of his extended observations on the living Echinoderm egg. His assumption of extruded cytoplasmic filaments of the *Asterias* egg which serve to draw the blunt-nosed spermatozoa through the surrounding jelly to the surface of the egg had been largely discredited until fully substantiated many years later not only for *Asterias* but also for many of the *Asteroidea*. Even in regard to the quadrille des centres described by Fol in his paper of 1891 and attacked by Wilson and Mathews (1895), Fol had a case. In his 1879 paper (p. 210) Fol remarked that in heavy polyspermy the sperm asters assume identical distances from one another placed with their centers along a theoretical circle. This fits in with the findings of E. L. Chambers (1939) regarding the sperm aster as a growing spherical gelled body. Several sperm asters simultaneously growing in size would assume the positions ascribed to them by Fol. Such symmetrical positions of four sperm asters would explain Fol's quadrille des centres. It was unfortunate that Fol was not able to correct his one wrong hypothesis because of his untimely death soon after publication of his paper.

terms of physical changes in the cytoplasm. He showed that the movements could be ascribed to the growing sperm aster as a gelled body (R. Chambers, 1917), the sperm pronucleus lying in or close to the center of the aster. The progressive increase in size of the aster transfers the sperm pronucleus passively to a central position in the egg, while the egg pronucleus is carried to the sperm pronucleus by centripetal streaming in radial channels converging at the center of the aster. Fol (1879, pp. 105 and 194), who first described the aster, had already presented the idea that the astral radiations are due to streams of centripetal flow.

The normal dissolution of the germinal vesicle of the fully grown oocyte initiates a gradual and prolonged process (R. Chambers, 1921) which converts the somatic cytoplasm of the ovarian egg into the karyocytoplasm of the maturing egg ready for fertilization. The experiments described in this paper, a brief account of which has been published (R. Chambers and E. L. Chambers, 1940), present the matter in detail with evidence concerning hitherto unsuspected causal interrelations between the egg nucleus, the sperm pronucleus, and the egg cytoplasm during and after alteration of the cytoplasm by the spontaneous dissolution of the germinal vesicle. These interrelations constitute, as it were, the performances of a three ring circus in the maturation of the egg.

The experiments stress features which are concerned with the egg and sperm nuclei during their earlier stages before the sperm aster has attained full expression. They are not to be compared with the egg fragmentation studies of Delage (1899), Tennent, Taylor and Whitaker (1929) and Whitaker (1928), all of which were done on fully mature sea urchin eggs and with reconstituted female pronuclei, both polar bodies already having been eliminated.

MATERIAL AND METHODS

The starfish egg is admirably suited for the present study, since, commencing with the germinal vesicle stage, the eggs develop in sea water and insemination can take place at any time.

Fol (1877) had observed that the eggs of *Asterias glacialis* normally are expelled into the sea water with the germinal vesicle still intact. In our work the fully grown germinal vesicle eggs were uniformly obtained by removing the ripe ovaries into finger bowls of sea water, where the eggs were immediately distributed in a large volume of sea water. Most of the work was done during the months of June and July. Only those batches of eggs were used in which over 90 per cent of samples of the eggs matured. All the bisecting operations on the eggs were done under oil and water immersion objectives.

The fragmented eggs and their controls were maintained at a temperature of 16° C. in syracuse watch glasses. The operations and observations were made in hanging drops suspended from a coverslip in the moist chamber of a micro-manipulator at room temperature. The eggs were transferred to the moist chamber, and several eggs immediately bisected. This required about three or four minutes. The eggs were then replaced in the watch glasses at 16° C., kept there until a few minutes before appearance of the sperm aster was expected, and then re-transferred to the moist chamber for observation.

The bisections were performed on the eggs at varying intervals after dissolution of the germinal vesicle, some before and others after insemination. The eggs,

suspended from the roof of the moist chamber, were divided by compressing them with the horizontal shaft of a slender microneedle. The vitelline membrane of the unfertilized egg and the enveloping membrane of the fertilized egg are firm enough to remain more or less intact during the bisection. The two egg fragments, which immediately round up and are completely separated, tend to remain together. The cutting was generally done so as to have both fragments of about the same size, one fragment never being smaller than about one half the volume of the other. Such a difference in size had no appreciable effect on the time of appearance of the polar bodies or of the sperm aster. This is in accord with Tennent, Taylor, and Whitaker (1929) who had shown that the cleavage time of egg fragments is independent of size as long as the fragments, when fertilized, undergo segmentation.

In all the experiments, every individual fragment was kept under observation simultaneously with its companion fragment in the same microscopic field. Hence, when a phenomenon was detected in one fragment it could be immediately compared with what might appear in the companion fragment. The time sequences and the phenomena looked for in each individual case were so clear-cut that intervals as short as two minutes were significant. The phenomena observed were the appearance in the granular cytoplasm of a diminutive radiating star which represented the sperm aster, and the elevation of a hyaline nipple on the surface of the egg, the beginning of one or other of the polar bodies.

Bisecting eggs with intact germinal vesicles confirmed the already recognized finding that fragments lacking the germinal vesicle are not fertilizable (Delage, 1901). After normal dissolution of the germinal vesicle, both fragments are capable of being fertilized, one with a diploid (sperm and egg), and the other with a haploid (sperm) nucleus.

The bisection of eggs already inseminated was done at varying times prior to first polar body formation. As was to be expected, only those fragments were capable of further development which contained the sperm pronucleus. Special attention was given to those eggs in which the sperm and egg nuclei were separated, one in each fragment.

RESULTS

The investigation is classified under two general headings. The first deals with observations on the sequence of events in whole eggs, and the second with bisected eggs. In the latter, attention was directed toward the reactions of the male and female nuclei when together and when isolated in the respective fragments of karyocytoplasm.

I. Observations on the Whole Egg

A. The unfertilized egg

The first intimation of the dissolution of the germinal vesicle is the development of an irregular contour of the membrane and a fading from view of the prominent nucleolus. An irregularity in shape of the membrane is not necessarily related to impending dissolution of the germinal vesicle. A mere collapse of the membrane induced by shaking the eggs does not accelerate maturation. The one

visible change which consistently heralds dissolution is the disappearance of the nucleolus. This is followed by disappearance of the nuclear membrane and a diffusion of the nucleolar contents mixed with the hyaline karyoplasm of the nucleus into the granular cytoplasm. Within five to fifteen minutes, the region formerly occupied by the germinal vesicle is filled with cytoplasmic granules indistinguishable from the rest of the egg. In the granular cytoplasm it is possible to detect the diminutive, hyaline egg nucleus which later gives off the polar bodies.

TABLE I

Sequence of events in the maturing unfertilized eggs of Asterias forbesii at 16-18° C.

	Time
From time of deposition in sea water to:	
Disappearance of nucleolus (50% completion)	8'- 19'
Formation of 1st polar body (50% completion)	76'- 90'
Formation of 2nd polar body (50% completion)	105'-119'

Table I gives the approximate times of the three most obvious events during the maturation of the unfertilized egg. The data were obtained from ten separate batches of eggs of at least 100 eggs in each, kept at a temperature of 16-18° C. The variations in the times recorded are due to the different batches. Within a single batch the variations did not exceed two to three minutes. The figures to the left denote the times, within a two minute range, recorded for seven of the batches. The figures to the right are of one batch. The times for the two remaining batches lie in between.

B. The fertilized egg

Table II, with data averaged from records of five batches of eggs, presents an analysis of the effect on the appearance of the first and second polar bodies and of

TABLE II

Effect of insemination on time of 1st and 2nd polar body formation, and of 1st cleavage in eggs of Asterias forbesii, at 16° C.

(1) Eggs inseminated at following inter- vals of time after deposition in sea water:	(2) Time 50% 1st P.B. formation after deposition in sea water:	(3) Time 50% 2nd P.B. formation after deposition in sea water:	(4) Time 50% 1st cleavage after deposition in sea water:	(5) Time 50% 1st cleavage after 2nd P.B. formation:	(6) Time 50% 1st cleavage after insemination:
—	78.2 (unfertilized)	107.3 (unfertilized)	—	—	—
25'	70.0	98.0	169.0	70.5	144.5
40'	71.5	101.0	172.0	71.0	132.0
50'	74.0	102.5	173.0	70.5	123.0
60'	75.8	104.0	175.0	71.0	115.0
70'	77.5	105.5	178.0	72.0	108.0
80'	—	106.5	184.5	78.0	104.5
90'	—	107.0	193.5	86.5	103.5
100'	—	107.5	203.5	96.5	103.5
130'	—	—	234.0	127.0	104.0

the first cleavage by inseminating the eggs at successive intervals following breakdown of the germinal vesicle. The first column gives the times of insemination. The figures in the second and third columns show that, up to a certain time, the earlier the insemination the more accelerated is the formation of the polar bodies. When the insemination is delayed to and beyond the time of first polar body formation, there is no evidence of acceleration, whereupon, the time of appearance of the second polar body tends to coincide with that of its appearance in the unfertilized egg.

Evidently it is only when the fertilization process is started early that the conversion of the egg nucleus into its pronucleus is accelerated. Later, when fertilization occurs at the time that the polar body formation has been initiated, there is no longer any appreciable accelerating action.

A consideration of the cleavage times, presented in the fourth, fifth and sixth columns, brings out several significant features. From the fourth column it can be seen, as is to be expected, that cleavage time corresponds with the time the eggs are deposited in sea water. However, during the earlier stages, up to some time before first polar body formation (after 60 minutes), the lapse is not as great as during the later stages (cf. Fig. 1). This is brought out more clearly from the figures in the fifth column which give the times between those of first cleavage and of second polar body formation. They indicate that the time interval, irrespective of insemination time, is constant until about the time when the first polar body is being initiated. After this the cleavage time becomes directly proportional to the insemination time.

The figures in the sixth column give the times between insemination and first cleavage. They show that the earlier the insemination up to the time when the first polar body is initiated (about 70 minutes), the longer is the time which elapses before cleavage occurs. After 70 minutes the time between insemination and cleavage becomes constant.

These analyses indicate that the rate at which the fertilization events proceed depends upon the cytoplasmic maturation which is completed at about the time of first polar body formation. Prior to this, it would seem that the immature state of the karyocytoplasm has a delaying effect on the development of the sperm and its accompanying events. Upon initiation of first polar body formation, the maturation of the karyocytoplasm is complete, whereupon the development of the sperm from the time of its entry proceeds without delay and cleavage occurs within a constant period of time.

A graphic presentation of Table II is given in Figure 1. The abscissae represent the times of insemination; the ordinates, the times when the various events occur. Concerning the unfertilized egg, the two vertical dotted lines and the two horizontal dotted lines intercept the X and Y axes respectively at the times when the first polar body forms (average of 78.2 minutes) and when the second polar body forms (average of 108.2 minutes).

Concerning the fertilized egg, the three solid curves represent the times for the formation, respectively, of the first and of the second polar bodies, and of the first cleavage in eggs inseminated at different intervals after germinal vesicle breakdown. The curves for the first and second polar body formation are parallel throughout and their upward slopes represent the acceleration due to insemination.

It is to be noted that when the insemination occurs at 78 minutes (time of first polar body formation) or later, the time of second polar body formation remains the same as that of the unfertilized egg.

Let us now consider the dotted dash curve which represents the time of first appearance of the sperm aster and which was calculated from data obtained on about 100 eggs observed with an oil immersion objective. The sperm aster never appears until after the second polar body, no matter how early the eggs have been inseminated (the earliest recorded being at 25 minutes). During these earlier

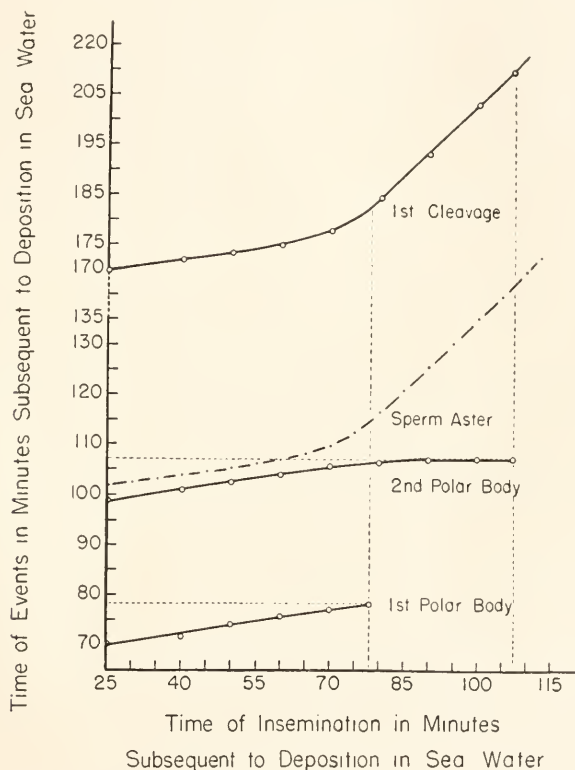


FIGURE 1. Relation of time of insemination to time of appearance of first P.B., second P.B., and first cleavage (solid lines), and of sperm aster (dotted dash line). Faint dotted horizontal lines represent times of appearance in unfertilized eggs of first and second P.B. The observations were made on eggs of *Asterias forbesii* at 16° C.

stages of the developing egg, it appears at two to three minutes after the second polar body has been given off. In later stages, viz., after 70 minutes, this interval becomes progressively greater. It is to be noted that the interval between the time of appearance of the sperm aster and the time of first cleavage is always just about 68 minutes, whether the time of insemination is early or late. This is the normal time for the events following aster appearance. The length of the interval between the time of insemination and the time of sperm aster appearance varies, depending upon the state of maturation of the karyocytoplasm. It becomes con-



stant only when the egg is fully mature, which also coincides with the time of first polar body formation. Thus, when the eggs are inseminated as early as 25 minutes after being placed in sea water, a period of about 78 minutes must elapse before the sperm aster appears. When the egg is inseminated during or after the first polar body formation, the interval between insemination and appearance of the sperm aster is found to be constantly about 35 minutes.

Insemination after formation of the first polar body results, as Lillie (1915) has shown, in a tendency toward a decline of fertilizability and of subsequent development. Abnormalities become pronounced when eggs are inseminated 30–60 minutes after the formation of the second polar body.

To summarize: there is a period of progressive ripening of the karyocytoplasm. Optimum ripening is heralded by the development of the polar asters and the initiation of the sperm pronucleus to form its aster. After this period there is a decline in the proper functional interrelations between the sperm and karyocytoplasm. The decline is made evident by the fact that sperm entry, subsequent to second polar body formation, results in an increasing abnormality of cleavage.

II. Observations on Bisected Eggs

A. Unfertilized eggs bisected and the fragments immediately inseminated

1. **Early bisections up to about ten minutes before first polar body formation.** Thirty pairs of fragments were studied. The cutting was done at 25, 40 and 60 minutes after deposition of the eggs in sea water (ca. 10, 25 and 45 minutes respectively after the germinal vesicle had disappeared). Each pair of fragments was then inseminated immediately. Figure 2 is representative of all the cases. The sperm aster in the non-egg-nucleated fragment appeared earlier than in the egg-nucleated fragment. Its time of appearance was always *after* the companion fragment had formed its first polar body and two to three minutes *before* the formation of the second polar body. On the other hand, in the egg-nucleated fragment, the sperm aster never appeared until two to three minutes after the second polar body had been formed. This difference between the two fragments was reflected in the earlier cleavage of the haploid fragment.

2. **Late bisections immediately before and during first polar body formation.** Twenty pairs of fragments were studied. In all of them the sperm aster appeared simultaneously at about two to three minutes after formation of the second polar body in both non-egg-nucleated and egg-nucleated fragments of each pair. The cleavage time of both fragments was simultaneous.

B. Eggs fertilized early and fragmented at varying times until shortly after first polar body formation

The eggs were inseminated 25 minutes after deposition in sea water, that is, shortly after dissolution of the germinal vesicle. In many of the bisected eggs both male and female nuclei lay in the same fragment. These double nucleated fragments, regardless of the time of cutting, behaved exactly like the whole eggs in regard to the time of polar body formation, appearance of the sperm aster and subsequent cleavage. Attention was devoted to the few fragments in which the

sectioning had separated the sperm from the egg nucleus. Four fragments were of eggs cut at 35 minutes; three at 50 minutes, and six at 74 minutes after deposition in sea water.

The results are shown in Figure 3. In the eggs (A_1) cut 35 minutes after deposition in sea water, the sperm aster (A_2) appeared after the first polar body of the companion fragment and two to three minutes before the second polar body. In an egg (B_1) cut at 50 minutes, the sperm aster (B_2) appeared simultaneously with the second polar body of the companion fragment. In an egg (C_1) cut at 74 minutes, the sperm aster (C_2) appeared after the second polar body in the companion fragment. D represents the first cleavage stage, at 170 minutes, of the eggs, A, B, and C. Cleavage occurred, as is to be expected, only in the fragment containing the sperm pronucleus. The egg nucleus in the other fragment produced the first and second polar bodies at the same rate as that of fertilized control whole eggs and, finally, moved to a central position in the fragment, where, as the female pronucleus, it enlarged somewhat but otherwise remained quiescent. The time of appearance of the polar bodies was thus seen to be the same, irrespective of when the sperm pronucleus had been separated from the egg nucleus by the cutting process. Evidently neither a brief nor a long sojourn of the sperm pronucleus in cytoplasmic continuity with the egg nucleus affects the hastening which the fertilization process induces in the formation of the polar bodies.

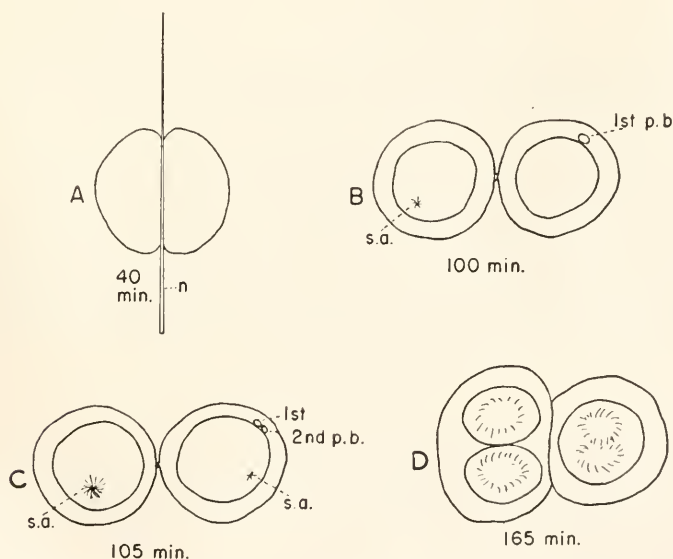


FIGURE 2. *Asterias* egg bisected 40 minutes after deposition in sea water, both fragments inseminated simultaneously.

A. At 40 minutes. Cutting of unfertilized egg with microneedle, *n*.

B. At 100 minutes. Both fragments with fertilization membranes. Haploid fragment with sperm aster, *s.a.* Diploid fragment with first P.B. which had formed 25 minutes earlier.

C. At 105 minutes. Haploid fragment with considerably enlarged sperm aster. Diploid fragment with beginning sperm aster and second P.B. which had formed two minutes earlier.

D. At 165 minutes. Haploid fragment just after completion of first cleavage. Diploid fragment still in amphiaser stage.

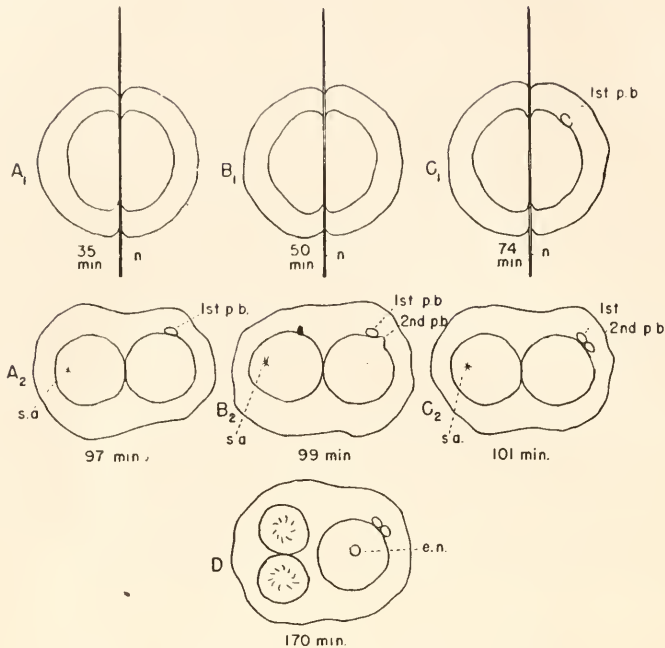


FIGURE 3. Three eggs inseminated 25 minutes after deposition in sea water and then bisected at different times so as to have sperm pronucleus in one fragment and egg nucleus in the other.

A. Cut at 35 minutes (10 minutes after insemination), A_1 . At 97 minutes, A_2 . Beginning sperm aster, *s.a.*, appears in one fragment and first P.B. in companion fragment.

B. Cut at 50 minutes (25 minutes after insemination), B_1 . At 99 minutes, B_2 . Beginning sperm aster appears in one fragment and beginning second P.B. in companion fragment.

C. Cut at 74 minutes, just after the first P.B. has formed, C_1 . At 101 minutes, C_2 . Beginning sperm aster appears in one fragment and completed P.B. in companion fragment.

D. Condition of all three bisected eggs at 170 minutes. Sperm-haploid fragment has cleaved, while egg nucleus, *e.n.*, of companion fragment has taken a central position and remained inactive.

DISCUSSION

The results presented in this paper stress two major features concerning the events after the material of the germinal vesicle has mixed with the cytoplasm of the egg. One deals with the maturation of the karyocytoplasm; the other, with the fertilization process of the male and female nuclear elements in their relations to the maturing karyocytoplasm.

Delage (1899) had already surmised that the dissolution of the germinal vesicle is essential to maturation and fertilizability of the sea urchin egg, and confirmed it from his merogonic experiments (1901) on the immature eggs of *Asterias glacialis*.

In our experiments with *Asterias forbesii* the disappearance of the germinal vesicle and the mixing of its hyaline fluid with the granular cytoplasm lasts about 10–15 minutes. The resulting karyocytoplasm contains the definitive egg nucleus.

Any viable fragment of this karyocytoplasm is fertilizable. The significant feature is that the karyocytoplasm must undergo a protracted maturing process. The prime evidence for completed karyocytoplasmic maturation is the appearance of asters initiated in the egg either by the egg nucleus in forming the polar bodies, or by the spermatozoon in forming its sperm aster.

It is of interest to note that when the fertilization process is initiated in eggs with still maturing karyocytoplasm, the activity of the egg nucleus starts *earlier* than it would if the egg were unfertilized.² On the other hand, the activity of the sperm pronucleus in forming its aster starts *later* when it is associated with the egg nucleus than it does when it is isolated in a separate body of karyocytoplasm. This indicates that the fertilization process accelerates the egg nucleus to polar body formation, while the presence of the egg nucleus delays the formation of the sperm aster. As an example of this, let us consider the situation in which an egg nucleus and a sperm pronucleus are lying together in karyocytoplasm which is still maturing. Upon completed maturation of the karyocytoplasm, there is initiated around either the male or the female nucleus a localized, centripetal cytoplasmic streaming which becomes evident to the eye as asters. The first asters to appear are those of the polar spindles of the egg nucleus. Not until the second polar body has been eliminated is there any sign of cytoplasmic streaming centered about the sperm pronucleus for the formation of the sperm aster. The course of these phenomena is of phylogenetic interest, viz., the fact that it is the egg nucleus rather than the sperm around which the radial streaming first occurs.

In the development of the sex elements, the last step taken by the fully grown primary oocyte is to undergo two successive cleavages (equational and reductional). In the early history of sex the resulting four egg cells may be equal in size or, in accordance with later evolutionary changes, they may be unequal, viz., the typical egg and its three polar bodies. In either event, growth of the mother cell, followed by two successive nuclear mitoses, has been repeated presumably over countless periods of time before the male sex cell came into being. This would establish a condition such that the maturation of the karyocytoplasm tends to lead directly to the formation of the two polar bodies. The sperm in the egg is a relatively late comer in evolution so that reactions concerned with it should come after, with the development of the sperm aster and eventually the amphiaser of the first cleavage spindle of the fertilized egg. This might be regarded as a case of evolutionary memory, colloidal or otherwise.

When the sperm pronucleus is isolated in a non-egg-nucleated fragment of an egg with karyocytoplasm which has not yet become mature, maturation leads to cytoplasmic streaming and aster formation about the sperm pronucleus. There is no egg nucleus to assert priority, and the result is that the sperm aster appears before it otherwise would.

When insemination occurs after the karyocytoplasm has completed its maturation, the conditions which now exist do not call for an interplay of the reactions described above. In a completely mature egg, the lapse of 30 to 35 minutes be-

² Recently, Lovelace (1947) was able by artificial means to accelerate the penetration of the spermatozoon in the *Nereis* egg. She found that this induced earlier formation of the polar bodies than would have been the case if the sperm had penetrated later. Fol (1879, pp. 117 and 335) had already noted that for the *Asterias* egg, polar body formation is accelerated by early insemination of the egg.

tween sperm entry and appearance of the sperm aster is just about the time between the initiation of the first and completion of the second polar body. Therefore, if sperm entry occurs at the earliest moment of completed karyocytoplasmic maturation, i.e., just prior to the formation of the first polar body, the astral streaming of the polar body spindles will have been completed before the sperm aster begins to be appreciable.

An indication of the necessity for proper time relations between the formation of the sperm aster and that of the polar body asters is given in extremely interesting experiments performed years ago by A. Brachet (1922). Brachet discovered a means of disturbing these time relations, and by doing so secured abnormal astral configurations. Brachet found that the immature eggs of *Paracentrotus*, on being removed from the ovary, could be stopped at various stages of their maturation by plunging the eggs into sea water. In the sea water these eggs readily became polyspermic, and the sperm which had entered continued to develop and formed sperm asters. These asters either remained small or grew to larger dimensions according to the stage of the eggs they were in. The stages of special interest in this discussion were those of the eggs possessing egg nuclear polar spindles and their asters. Sperm asters present at the same time became intermingled with them and formed abnormalities such as tripolar mitoses, etc. Fol was also able to observe similar discrepancies in polyspermic *Asterias* eggs. In the event that several sperm asters appeared while the chromosomal vesicles of the egg nucleus in mitosis were still infused, Fol noted that one or more of the vesicles became incorporated in the sperm asters, thus upsetting the normal course of events.

The avoidance of such a phenomenon is ensured in the *Asterias* egg, which normally matures in sea water and which is fertilizable at any stage during its maturation. In monospermic eggs an appropriate time-spacing between the male and female nuclear events is occasioned by the following: On the one hand, the formation of the egg-nuclear polar bodies is accelerated by the fertilization process, while on the other, the appearance of the sperm aster is delayed by the presence of the egg nucleus. The two features combine to separate in time the formation of the polar bodies from the formation of the sperm aster. The result is that in the normal course of development, the cytoplasmic streaming, involved in the formation of the polar body asters, reaches completion before the initiation of the streaming associated with the growing sperm aster. It appears, therefore, that the peculiar interrelations between karyocytoplasm, egg, and sperm nuclei are of service in preventing a possible interference between the reactions concerned in polar body formation and those concerned with preparation of the fertilized egg for its first cleavage.

SUMMARY

Full-sized germinal vesicle oocytes of *Asterias forbesii* undergo normal maturation in sea water. At 16° C. the first polar bodies are formed in about 80 minutes, and the second, in 108 minutes. The eggs are sperm-fertilizable from the time of germinal vesicle breakdown until some time after elimination of the second polar bodies. Fol (1879, p. 204) indicated that the optimum time for insemination is after germinal vesicle breakdown up to the first polar body formation. In accordance with Fol, the earliest period for the sperm aster to appear was found to be

always a few minutes after the formation of the second polar body. A feature to be stressed is the progressive change of the karyocytoplasm induced by the mixing of the contents of the germinal vesicle with the cytoplasm during maturation.

Maturation of the karyocytoplasm

1. When eggs are inseminated two to three minutes before first polar body formation or later, the time for the sperm aster to appear in the living egg is about 35 minutes at 16° C.

When the eggs are inseminated at any time prior to the above, the time taken for the sperm aster to appear is equal to 35 minutes plus the interval between the time of insemination and the time of initiation of the first polar body.

Evidently, therefore, the time of appearance of the sperm aster is a function of the maturation of the karyocytoplasm. The maturation begins at the time of germinal vesicle breakdown and reaches completion two to three minutes prior to formation of the first polar body. In a fully mature karyocytoplasm the interval between sperm entry and the appearance of the sperm aster is constant.

The egg nucleus

2. Sperm-fertilization of whole eggs or of egg-nucleated fragments accelerates the egg nucleus in the formation of its polar bodies. The earlier the insemination the greater is the acceleration.

3. The effect of the fertilization process in accelerating polar body formation persists after removal of the sperm pronucleus. This was ascertained by removing the sperm pronucleus, through bisection, at several intervals of time, the earliest being ten minutes after insemination.

In other words, once given the impetus the egg nucleus maintains its hastened progress independently of the presence of the sperm pronucleus.

The sperm aster

4. In eggs bisected while undergoing maturation and then inseminated, the sperm aster appears earlier in the fragment lacking the egg nucleus than in the egg-nucleated fragment.

In eggs fertilized while undergoing maturation and then bisected at different times, the sooner the sperm pronucleus has been isolated from the egg nucleus, the earlier the sperm aster appears.

In other words, the presence of the egg nucleus has a delaying action on the development of the sperm aster. However, the earlier the egg nucleus has been removed through bisection of the egg, the less is the delaying action.

GENERAL CONCLUSION

There is a close interrelation between (a) the fertilization process, (b) the ripening of the karyocytoplasm, (c) the development of the sperm pronucleus, and (d) the activity of the egg nucleus in forming its polar bodies. The fertilization process, by hastening the maturation of the karyocytoplasm, accelerates the activity of the egg nucleus in forming its polar bodies. On the other hand, the egg nucleus

exerts a lag effect on that feature of the maturation of the karyocytoplasm which is concerned with the development of the sperm aster. The net result is the attainment of an adequate spacing between the times of the cytoplasmic streaming activities concerned with polar body formation and those concerned with the development of the sperm aster. This permits normal development of *Asterias* eggs fertilized at any time during their maturation.

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FORM AND GROWTH IN THE DEVELOPMENT OF A SCYPHOMEDUSA

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The nature and development of the scyphistoma and strobila of certain Scyphomedusae have been described a number of times, from various points of view.

Among the Semaestomae, our knowledge of Aurelia and Chrysaora (including Dactyometra) is fairly complete, although correlations of form and size have not been emphasized. The other two forms that have been studied to some extent are Pelagia, the egg of which transforms directly into a medusa, and Cyanea. In the case of the Rhizostomae the developmental cycle is known for Cassiopea, Cotylorhiza and Nausithoe.

The present account is based upon a collection of scyphistomae and strobilae tentatively identified as those of *Cyanea capillata* Eschscholtz.

SOURCE OF MATERIAL

The material was part of an unlabelled collection in the Zoology museum at McGill University, a circumstance that adds an uncertainty of original site to the usual uncertainty of parentage of scyphistomae found in their natural habitat.

Fortunately, the internal evidence is decisive. The scyphistomae were attached to ascidians or to eel grass (*Zostera marina*) to which the ascidians in turn were attached. Fastened between some of the ascidians were several very young specimens of *Cucumaria frondosa*. The presence of the holothurian places the locale on the Atlantic coast north of Cape Cod. The ascidian is definitely identified as *Molgula provisional* Van Name, a species closely related to *M. manhattensis* and previously confused with it (cp. Van Name, 1945, p. 389). *Molgula provisional*, however, is recorded only from waters in the general region of Eastport, Maine, from Passamaquoddy Bay to Mount Desert. Since it is known that collections of this species of *Molgula*, attached to eel grass, have been made at St. Andrews Point in Passamaquoddy Bay, there is little doubt that the material is part of such a collection, and in any case there appears to be no doubt that these scyphistomae came from shallow water near the mouth of the Bay of Fundy.

IDENTIFICATION OF MATERIAL

Identification of the genus and species is rather more difficult. The obvious suspects are *Aurelia aurita* and *Cyanea capillata*, since both of these are abundant in the region. *Dactyometra quinquecirrha* (a "Chrysaora") reaches the shoreline at Cape Cod, but is not reported from inshore waters of northern New England. The most northerly occurring rhizostomid of the Atlantic coast is *Rhopilema verrillii*, a southern form that occasionally strays into Long Island sound. The only

remaining form is *Phacellophora ornata*, another semaeostomid, which is known only from Eastport and the Bay of Fundy as two isolated records, by Verrill in 1869 and Fewkes in 1888.

The strobilae do not resemble those of *Aurelia* (cp. Percival, 1923) or *Chrysaora* (cp. Chuin, 1930), and while they are remarkably like those of the rhizostomids *Cassiopea* (cp. Bigelow, 1900) and *Cotylorhiza* (cp. Claus, 1892), it is not reasonable to assume the occurrence of an unknown rhizostomid in the region in question, nor to extend the range of *Rhopilema* from Long Island Sound through the five hundred miles of cold water north of Cape Cod. The alternatives remain *Cyanea* or *Phacellophora*, and the absence of any record of *Phacellophora* during the last sixty years makes it a most unlikely candidate. It is provisionally assumed, therefore, that our scyphistomae and strobilae belong to *Cyanea*, even though the somewhat brief earlier descriptions of the life cycle of *Cyanea* are significantly different from the account given here.

The *Cyanea* of the western Atlantic is *C. capillata* Eschscholtz. According to Mayer (1910), *C. arctica* Perón and Lesueur and *C. lamarckii* Perón and Lesueur are synonymous, or at the most are varieties of doubtful stability. The embryonic and early larval stages have been intensively studied by Hyde (1894) as *C. arctica*. Young scyphistomae were reared by L. Agassiz (1862) as *C. arctica* and by Perez (1920) as *C. capillata*. Planulae were reared in aquaria through the scyphistoma to the strobila and ephyra stages by Hargitt (1902 and 1910) as *C. arctica* and by Delap (1905) as *C. lamarckii*. The scyphistomae described by the above investigators might well be of one and the same species, but the strobilae are very differently described and in neither case do they conform at all closely with the one given here. Both Hargitt and Delap obtained planulae directly from known medusae, and the difference expressed in their descriptions must be due either to differences in culture conditions or to a genetic difference in the parent organisms. These differences will be discussed following the description of the present material, which in spite of the element of doubt will be assumed to be that of *Cyanea capillata*.

GROWTH OF THE SCYPHISTOMAE

Since there is no indication that long lateral stolons are formed, as in *Aurelia*, that could produce buds at a considerable distance from a parent scyphistoma, the minute individuals found in scattered and very isolated positions are assumed to be newly attached planulae. The possibility of migratory buds, however, is not excluded. Typical examples are shown in Figure 1, A-D.

The planula apparently attaches by its narrow end, and in some cases at least sends out two or three root-like processes of attachment (Fig. 1, A, B). Four tentacles appear around the developing manubrium, while four more are added, bringing the number to eight without significant change in size from the original state (Fig. 1, C, D). Eight new tentacles appear, raising the total to sixteen, again with little increase in the size of the whole.

At the same time a small bud protrudes from the wall of the hydroid at or near the junction of the body and stalk (Fig. 1E). Similar buds, appearing at the same site, occur in scyphistomae of all sizes (Fig. 1, G-J), although many scyphis-

tomae equally representative of all sizes were found without buds (Fig. 1F). The conclusion is that a series of such buds may be produced by an individual scyphistoma. The first appearance of a bud in a minute scyphistoma is in itself an expression of a local acceleration of growth, and it would be gratuitous to assume that this growth would become abruptly arrested and that the same bud would remain

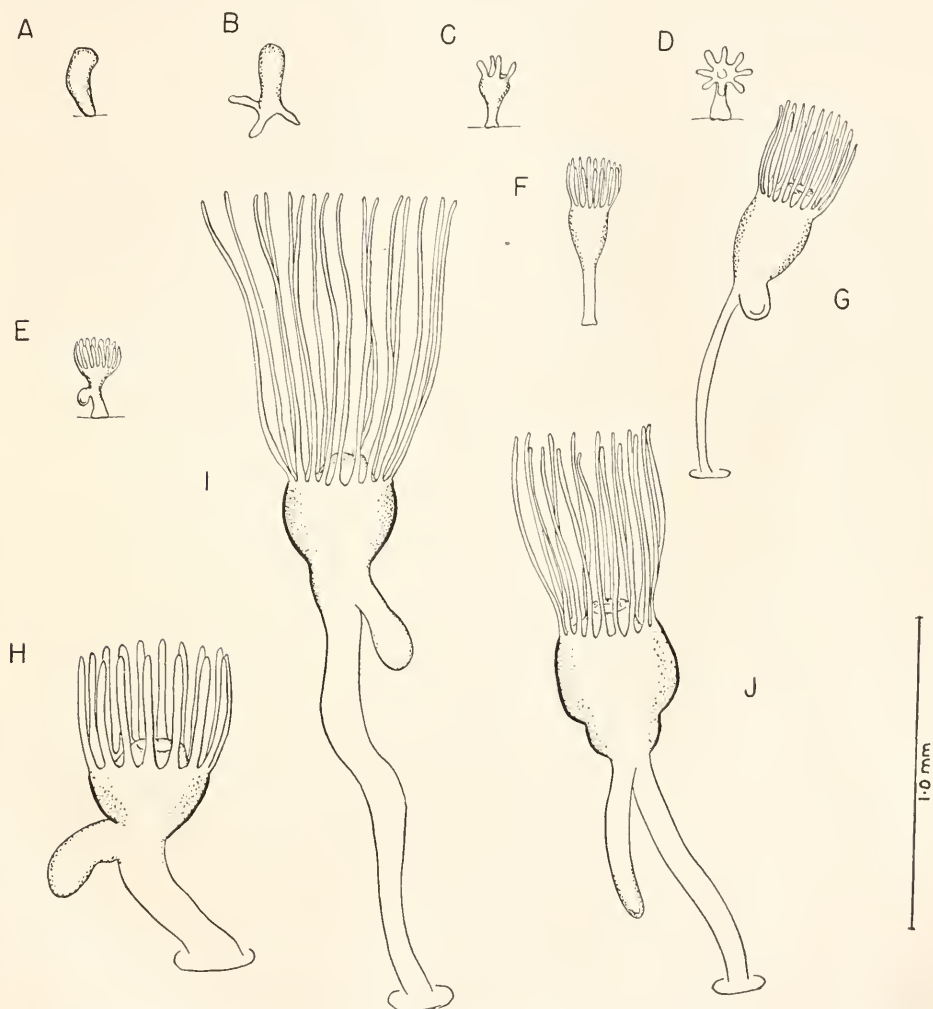


FIGURE 1. Growth and budding of scyphistomae of *Cyanca capillata*. A, B, attached planulae. C, 4-tentacle scyphistoma. D, 8-tentacle form. E, 16-tentacle scyphistoma with lateral bud. F, larger form without bud. G, H, I, J, older scyphistomae with buds.

but little changed in relative proportions in the large scyphistomae. It is more reasonable to interpret the conditions illustrated as being either the production of several buds successively from one site, or the production of but one bud, though at different stages of growth among different individuals.

In the great majority, the direction of growth of the bud is from the top of the stalk downwards towards the substratum. Growth of the bud is primarily stolonial, and is mainly by terminal proliferation of cells (cp. Fig. 1J). The largest scyphistoma of this type is shown in Figure 2B. No indication that such outgrowths extend to any distance has been found, and the occurrence of associations such as that shown in Figure 1, A and E, suggests that the buds grow down to become attached to the substratum close to the base of the parent, and constrict off from the parent at the point of origin.

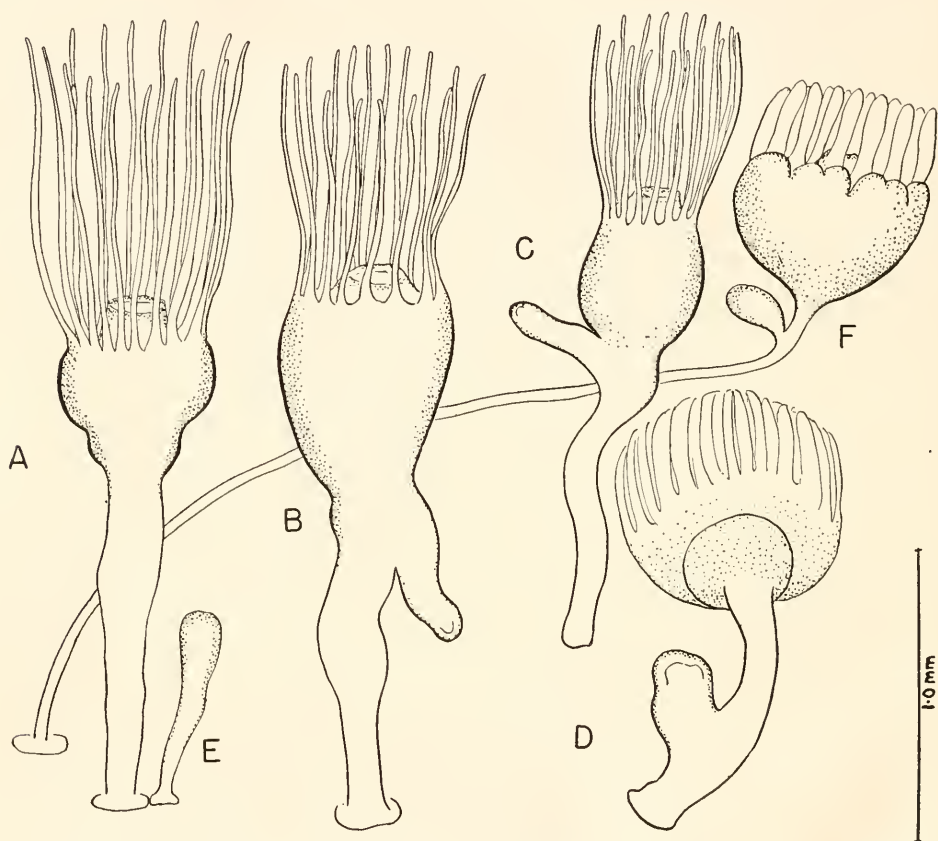


FIGURE 2. Fully grown scyphistomae of *C. capillata*. A, commencement of strobilation B, with bud directed downwards. C, D, with buds directed anteriorly. E, detached and attached bud at base of parent. F, metamorphosing scyphistoma with late bud.

In a minority of cases the bud grew upwards instead of downwards (Fig. 2, C, D) and in one case grew from the top of a long tenuous stalk that was bearing a metamorphosing scyphistoma at its end. Conditions such as these probably lead to those shown in Figure 3, A and B. In fact, Figures 2C and 3A might well be placed in sequence, the scyphistoma of Figure 2C having partially metamorphosed to become an ephyra in Figure 3A, the bud of Figure 2C having become a scyphistoma in Figure 3A, while the mutual relationship of the stalks remains unchanged.

On the other hand, the comparable stages of metamorphosis exhibited by the two heads of the individual shown in Figure 3B suggest the possibility that the division of the distal end preceded differentiation into scyphistomae, especially since the head that is somewhat the smaller is actually the more advanced, for only the eight interlobular tentacles remain. Such a condition seems more likely to arise

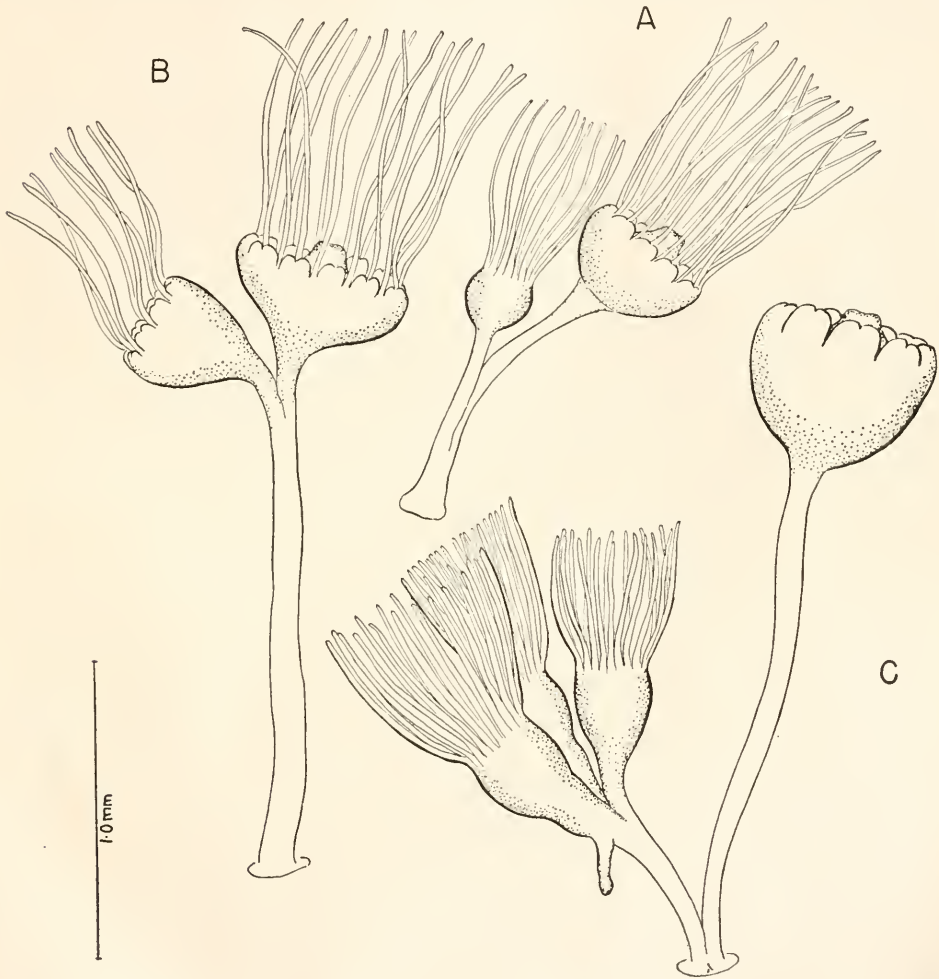


FIGURE 3. Retention and division of buds of *C. capillata*. A, bud forming scyphistoma attached to stalk of parent. B, double-headed strobila. C, strobila with three-headed scyphistoma attached to stalk base.

at the point of detachment of a bud from its parent than at the distal end of a newly attached planula. This is somewhat forcibly indicated by the example shown in Figure 3C. The parent scyphistoma is well advanced in its metamorphosis into an ephyra. The associated stalk may possibly have arisen from a bud similar to that seen in Figure 2D, but one arising even more proximally, or equally, if not more

likely, from a bud that grew downward from the usual site to become attached at the base of the parental stalk. In any case its distal end has given rise to three scyphistomae of approximately equal size. It does not seem possible that any one of the three could have given rise to the other two by budding, for there is too close an identity of size and form. In one of the three individuals a bud is growing downward, almost like a regeneration of an additional stalk to compensate for the multiplicity of heads.

FORMATION AND DEVELOPMENT OF STROBILAE

During the process of growth, the scyphistoma becomes progressively differentiated into stalk and head as in Figure 11. In many cases metamorphosis into an ephyra occurs in a typical manner and purely as a monodisk. The head shortens and widens, eight of the sixteen tentacles resorb during the formation of the eight rhopalia, while somewhat later the eight interlobular tentacles are also resorbed. At the same time, the outer margin of the scyphistoma divides into eight lobes corresponding to the lappets of the future ephyra.

While in many cases a single ephyra may form from the head of a scyphistoma, in as many others, if not more, two or three ephyrae are produced in series. Whether one or more are to be formed is discernible from the contour of the scyphistoma before there is any other metamorphic indication, as in Figure 1J and 2A. In most cases, if not all, the interlobular tentacles are retained until shortly before the ephyra is set free (Fig. 3A). In no case have tentacles been seen in a developing ephyra that is second in line.

Three stages in the later development are illustrated by Figure 3, A, C, and E, representing the eight-tentacle stage (3A), all tentacles resorbed (3C), and the fully developed ephyra on the point of liberation (3C).

Cases such as the one shown in Figure 3B, in which two ephyrae are almost at the same advanced stage of development, suggest that the ephyra probably grows to a certain critical size, when its development is functionally complete and it is ready to be set free, even though greater differences in size may be more evident at an earlier stage (cp. Fig. 3A). The individual shown in Figure 3D probably represents a second ephyra, the first having been liberated, and the same may be true for the primary individual in Figure 2C. Otherwise there is considerable variation in the time or size at which all tentacles become resorbed.

In all of the individuals with ephyrae, shown in Figure 3, there is present a relatively small basal swelling at the junction with the stalk, suggestive of a third ephyra. Marginal lobes tend to develop, though not in relation to any particular size (cp. Fig. 3A, 7D), and it is possible that an ephyra would have developed. The fact, however, that no individual has been found with three unmistakable ephyrae in process of formation may mean one of two things; either the third effort remains abortive, or else the first ephyra is always liberated before the third is definitely established.

It is notable that these third attempts at annular growth usually bear short tentacles in the lobular position (e.g. Fig. 3, C and E), possibly indicative of the re-establishment of the scyphistoma state.

A number of isolated stalks were found, of the same size as the largest bearing ephyrae, which possessed four distal tentacles as in Figure 3F. These may rep-

resent a return to the scyphistoma condition as is generally the case in *Aurelia* and *Chrysaora*, giving rise to another crop of ephyrae at some later time. On the other hand no scyphistoma was found that had a fully grown stalk and a head with either eight or sixteen tentacles. In our opinion such stalks as that illustrated are merely the final differentiation of the residual stumps after the ephyrae have been liberated, and in this form they do not give rise to further generations.

DISCUSSION

The essentially monodisk character of strobilation just described is much more reminiscent of the strobilae of the rhizostomids *Cotylorhiza tuberculata* (Claus, 1892) and *Cassiopea xamanchu* (Bigelow, 1900) than the polydisk strobilation described for *Cyanea lamarckii* by Delap (1905) at Valencia, and much more extreme than that of *Cyanea arctica* as described by Hargitt (1910) from Woods Hole. The question arises whether the differences indicate different parentage or a varying response to different conditions of growth.

Both size and shape appear to determine the type of strobilation, and since there is the possibility that the type may vary greatly with external conditions, it may be well to exclude *Aurelia* as a candidate somewhat more definitely. In the first place, a freshly liberated ephyra of *Aurelia* has a relatively shorter manubrium, gastral filaments much more remote from the manubrial base, and less suggestion of inter-rhopalial tentacles, than the ephyra of our present form shortly before liberation. Secondly, the manner of budding of the scyphistomae is markedly different. If the choice lies between *Aurelia* and *Cyanea*, as it appears, there is little doubt that *Cyanea* is the parent form.

The growth of a scyphistoma up to the time of liberation of an ephyra is divisible into three phases. The first concerns the transformation of the planula into a 16-tentacle scyphistoma. This phase has been intensively studied in relation to the manner of origin of the stomach pouches and the order in which the tentacles arise. Neither of these features greatly concerns us here; our main interest lies in the manner of growth and budding of the scyphistoma, and in the strobilation to form ephyrae.

The second phase, the growth of the 16-tentacle scyphistoma, is associated with the production of buds. In both the rhizostomids, *Cotylorhiza* and *Cassiopea*, buds arise one at a time from the scyphistoma body wall above the apex of the stalk. The buds break free, are ciliated and free-swimming, but they eventually settle and become attached by their original outer end.

In the semaeostomids *Aurelia* and *Chrysaora*, buds are formed initially as lateral outgrowths from the body wall near the base of the scyphistoma. They grow out as stolons for a considerable distance before becoming attached (Fig. 5B) either to give rise to a new scyphistoma at the point of attachment, or to one or two scyphistomae at some place between origin and attachment. The connection with the parent is finally broken.

In our *Cyanea* the buds arise from a site equivalent to the point of origin in *Cotylorhiza* and *Cassiopea*, but grow longer and downward to become attached basally by the time separation from the parent takes place. In both types, however, the scyphistoma head grows from the upper end of the bud. It is therefore intermediate in character between that of *Aurelia* and *Cassiopea*. The three kinds

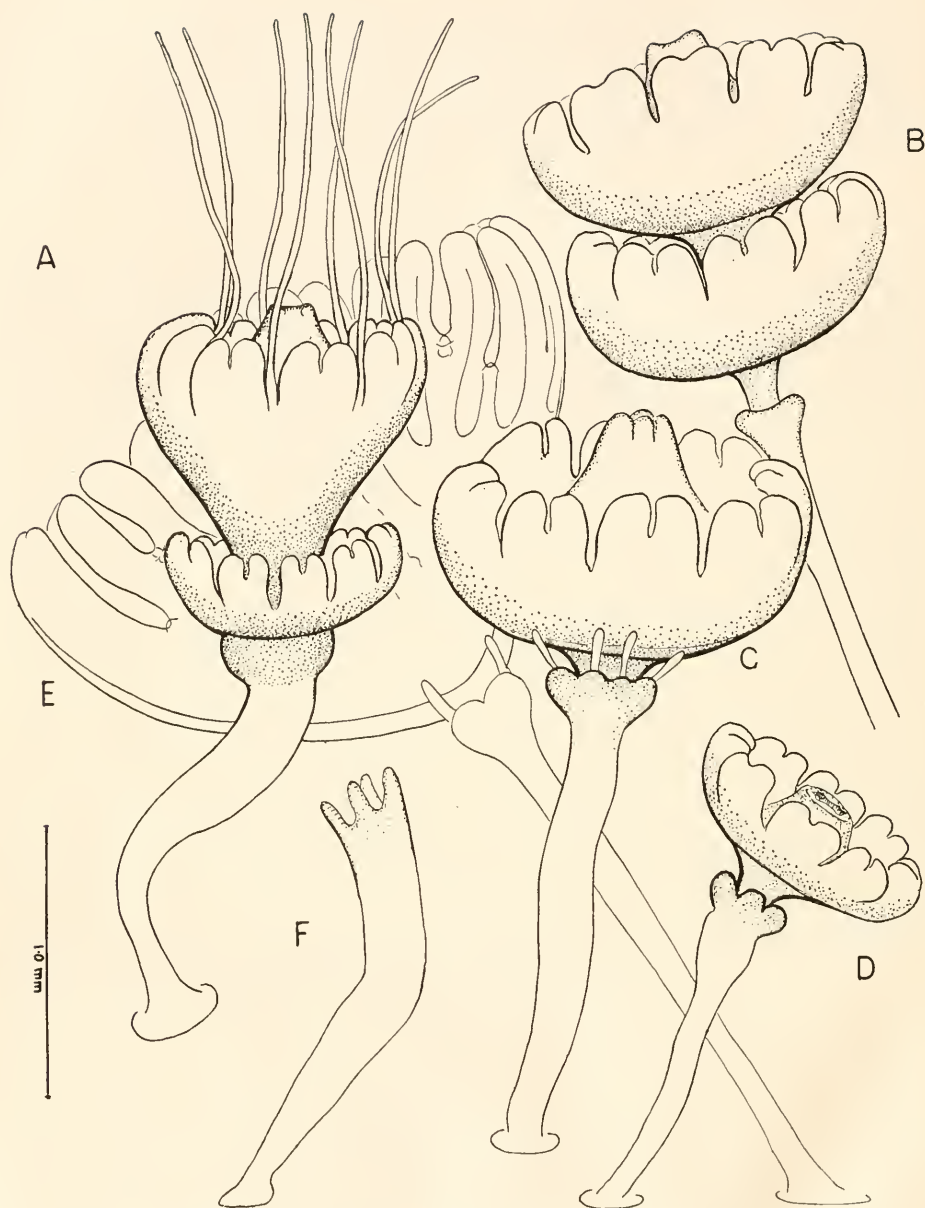


FIGURE 4. Strobilae of *C. capillata*. A, strobila with two ephyra and possible third. B, strobila with two equalized ephyrae. C, strobila with advanced ephyra and a potential second bearing scyphistoma tentacles. D, strobila with second ephyra well developed and a potential third. E, ephyra on point of liberation. F, post-strobila stalk with four tentacles.

of buds are essentially the outcome of two variables, the direction of outgrowth and the intensity of growth. Subsequent development depends upon the orientation of the outgrowth, and a new scyphistoma always arises from an upper surface, whether it be the distal or proximal end of an outgrowth or from some point on its side wall.

The question of monodisk or polydisk strobilation concerns both size and shape, both of which are expressions of growth. In monodisk development, growth in the basal part becomes progressively linear and apparently becomes arrested, while anterior growth becomes progressively transverse. Between the two regions there is a steep growth gradient producing a comparatively abrupt transition from head to stalk.

In contrast to this, the scyphistoma of *Aurelia* exhibits no such differentiation, and both transverse and linear growth occur throughout, so that while growth in length of the whole is the greater, transverse growth continues in basal as well as anterior regions. A large scyphistoma is therefore not very different in shape from a small one.

Shape is probably one of the main factors in determining the nature of strobilation. Constrictions carve off the shallow saucer-like discs of the scyphistoma to form ephyrae, and whether one, two, or many such discs can be produced is mainly a matter of the shape of the whole and the extent of growth occurring at the various levels. In this light, the difference between monodisk and polydisk strobilation is primarily a difference in the extent to which significant transverse growth can be maintained along the antero-posterior axis of the scyphistoma (cp. Fig. 2A, 5C). This activity may well vary with different conditions of temperature and food supply.

The scyphistomae reared by Delap grew steadily through summer months, apparently without producing buds, in each of two successive years, and in each year strobilated to form eight to eleven ephyra in late winter when the temperature fell below 45° C. The scyphistomae were abundantly fed with small planktonic organisms throughout the whole period. Those reared by Hargitt were fed even more concentratedly, at relatively high temperatures, and grew to the strobila condition with astonishing rapidity. One to five ephyrae were produced, with an average of three to four. Hargitt states that buds were seen but were extremely rare. Fortunately Delap gives the scale of her drawing of the strobila, so that a comparison of actual size is possible. Her polydisk strobilae are approximately three times the height of ours, and have no sharp division into stalk and head.

Our own scyphistomae were without doubt collected during the summer or late spring, and in Passamaquoddy waters would accordingly be developing at low temperatures (below 50° C.), even though maximum for the region. Growth would be relatively slow at the prevailing temperature and the food supply would probably fall far short of the degree of forced feeding employed by Hargitt and Delap.

The form of the sessile phase of the Hydromedusae responds sharply to varying conditions of temperature and food supply (Berrill, 1948, 1949) and it would be expected that the scyphomedusae would also react, in their own way. Differences in relative growth rates, however, may very well be inherited within the limits of a single species, and different races of *Cyanea capillata* may vary in the quantitative growth response their respective scyphistomae make to changing external conditions.

SUMMARY

The developmental cycle of a scyphomedusa, probably *Cyanea capillata* Eschscholtz, is described, with emphasis upon the correlation of size and form.

The nature of the budding process, giving rise either to free buds or to double-headed forms, is described.

An analysis of monodisk and polydisk strobilation is given in terms of growth, size and shape.

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FUNDAMENTAL PRINCIPLES IN OXIDATION-REDUCTION^{1, 2}

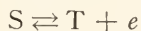
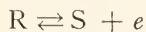
L. MICHAELIS

From the Laboratories of The Rockefeller Institute for Medical Research, New York

Oxidation of organic compounds is the source of energy for living organisms. This mechanism of supply of energy is made possible by the fact that the organic compounds used as food, as well as the oxidizing agent, molecular oxygen, are inert and can, at the proper time, be activated by catalysts so as to interact with each other. This inertia is due to the principle of "compulsory univalent oxidation" or of "single-electron transfer." This may be explained as follows. Oxidation is, primarily, the withdrawal of electrons. It is unessential for the process proper of oxidation whether a proton is withdrawn together with the electron. If so, oxidation is the same as dehydrogenation. Reduction is the reversal of oxidation. The principle just mentioned states that any bivalent (or polyvalent) oxidation or reduction has practically no other chance to proceed than in successive transfers of a single electron, or, in "univalent steps" of oxidations or reductions. These steps may overlap, and often to such an extent that the nature of the two-step process is difficult to recognize. In this way, "bivalent" oxidation or reduction of such substances as quinones and dyestuffs was conceived until recently as a bivalent process occurring by the simultaneous transfer of a pair of electrons.

The experimental evidence for the principle of single-electron transfer can be furnished essentially by two methods: measurement of redox-potentials, and measurement of magnetic susceptibility.

In a reversible redox system, if it is a univalent one, such as $\text{Fe}^{+++}\text{--Fe}^{++}$, the molecular species involved can exist on two levels of oxidation. If it is a bivalent one, according to the principle of single-electron transfer, it can exist on three oxidation levels: the reduced form, R; the semioxidized, S; and the totally oxidized, T, which are related to each other as follows (e is the electron):



The equilibrium $2\text{S} \rightleftharpoons \text{R} + \text{T}$ is always established with unmeasurably high speed, just as the equilibrium of electrolytic dissociation, in contrast to most other reactions in organic chemistry which usually are relatively slow, the rate being measurable and strongly dependent on temperature.

All valence-saturated organic compounds have an even number of electrons, each chemical bond being represented by an electron pair. So, any S compound

¹ Paper presented as part of a Symposium of the Society of General Physiologists, Woods Hole, September, 1948.

² This paper was originally presented with the aid of about fifty lantern slides representing experimental evidence and a few demonstrations of experiments. The abstract given here is made up in a form supposed to be understandable without this aid.

must have an odd number of electrons; it must be a free radical. Two molecules of a free radical may or may not combine to form a dimer, D, which again has an even number of electrons. In most cases, among organic dyestuffs of the type of methylene blue, or the flavine dyes of the yellow respiration enzymes, the intermediate form is a free radical, S, and not D. The existence of an intermediate form, be it S or D, can be easily recognized on oxidizing R or reducing T, because the intermediate form usually has a color of its own. Whether it is S or D can be recognized as follows. When a solution of R is titrated with an oxidizing agent, and the potential at a blank platinum electrode is plotted against the percentage of oxidation, the curve obtained in this plot is independent of the initial concentration of R, if the intermediate form is S; in contrast, its shape strongly depends on the initial concentration if the intermediate form is D. The experiment shows that in most cases there is S; in some cases, at higher concentration, some D may be in equilibrium with S. Furthermore, the equilibrium constant, called the semi-quinone formation constant

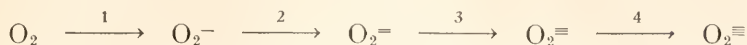
$$\frac{[S]^2}{[R] \times [T]} = k$$

can be measured with this method: The slope of the titration curve depends on k. One can calculate k from this slope. If k is very large (> 16) the titration curve is not simply S-shaped but shows a steepening around 50 per cent oxidation, which directly manifests the two steps of the oxidation.

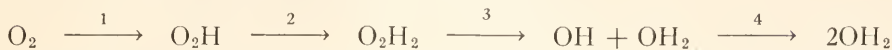
The magnitude of k depends on the chemical nature of the redox system and on pH. For cationic redox systems (say, basic dyestuffs) k increases with decreasing pH; sometimes the separation into two steps is clearly recognizable in extremely acid solution, e.g. in methylene blue. For anionic redox systems (such as quinone systems), k increases with increasing pH. However, k never becomes vanishingly small for reversible redox systems. The fact that S at any pH is capable of existence in a finite concentration, is the condition *sine qua non* for the reversibility of the redox system. If k is utterly small, it means that the S state is capable of existence only in infinitely small concentration. Since the oxidation has to pass through the S-state, it means that the rate of the oxidation or reduction is slow, that a high activation energy is required both in the direction $R \rightarrow T$ as well as $T \rightarrow R$. For instance, the oxidation of ethanol to acetaldehyde is irreversible and needs a high activation energy because the S form (which would be $\text{CH}_3\cdot\text{CH}\cdot\text{OH}$, a free radical with "tervalent" carbon), is utterly unstable. If this process has to be made reversible, as it is in the living organism, some means must be provided to increase the stability of the S form.

Another method of demonstrating a free radical, S, during the reduction of $T \rightarrow S$ is the measurement of magnetic susceptibility. Since the uncompensated spin of the odd electron in a free radical must produce paramagnetism, free radicals can be recognized, and their concentration determined, by the measurement of magnetic susceptibility. When the solution of a suitable quinone is slowly reduced by glucose in an alkaline solution, the magnetic susceptibility changes first in a direction indicative of the appearance of a free radical, later in a direction to indicate its disappearance again.

Not only does the oxidation of organic compounds as used for food need activation, but also molecular oxygen needs activation for its reduction. The successive steps of the reduction of O_2 are:



or, in presence of water, which can furnish protons:



The barrier for this reaction is represented by the high energy content of the radicals O_2H and OH . A high activation energy is required to reduce oxygen to hydrogen peroxide, and also to reduce hydrogen peroxide to water.

Enzymes concerned with oxidation-reduction exhibit their function in lowering the activation energy. The enzyme forms a reversible compound with the substrate. In such enzymes there are always two substrates: the electron acceptor (such as O_2 , or Fe^{+++} , or a flavine dye), and the electron donor (such as glucose or lactic acid). One of the two "substrates" represents a coenzyme or a prosthetic group such as heme. It is over and over again reduced and oxidized reversibly in the course of metabolism. The other "substrate" is the substrate proper. Although probably all oxidation-reduction processes are conducted in a reversible manner by enzymes, the whole process, as far as the "substrate" proper is concerned, goes one way only because the reaction product is immediately removed and shuttled on to another enzyme which will cause a further step in its metabolic change.

The problem as to how the enzyme brings about the lowering of the activation energy may be answered as follows. The attractive force exerted by an enzyme to its substrate, resembling that of the force between a protein and its immunological antibody, brings about the enzyme-substrate compound, and even the ternary compound consisting of apoenzyme (the protein part of the enzyme), prosthetic group and substrate proper. The specific shape of the protein surface forces the substrate molecule into a shape not attainable spontaneously. The energy released by the formation of the compound is not entirely dissipated as heat but used to distort the substrate in such a manner as to ease the making of the free radical which is the necessary intermediate step of the oxidation or reduction. The free radical does not exist, under these conditions, in a free state, but as an intramolecular constituent of the enzyme-substrate complex only. The case is comparable to the "activation" of hydrogen by platinum black. The attraction of Pt towards H_2 is strong enough to squeeze the H_2 molecule into the lattice of the Pt atoms in which it does not entirely fit. The H_2 is hereby stretched so that it behaves now almost as though it consisted of two H atoms. An H atom is the analogue of an S form; it contains an odd number of electrons, namely one. The difference is that the enzyme is specific, sometimes for the electron donor, sometimes for the acceptor, and often for both.

PLANT HORMONES, GROWTH AND RESPIRATION¹

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One of the greatest values of the discovery of the auxins as growth hormones in plants was that they made it possible to control growth. From the study of growth as produced under controlled conditions by auxins came a number of experiments on the interrelation between respiration and growth. Some of these, and the conclusions to which they lead, will be reported in this paper. Problems concerned with the rate of formation, use, and inactivation of auxin in the intact plant represent another field of endeavor which will not be considered herein. It should also be made clear at the outset that the precise chemical nature of the auxin of higher plants need not concern us here. Most of the experiments below were carried out with indole-acetic acid, which is a natural auxin of widespread occurrence in both higher and lower plants. For this type of work the auxin is regarded merely as a tool to produce growth at will.

Now the central problem in regard to auxin and the growth of plants is an old one; namely, how it is that one substance can produce many different kinds of effects. Visible growth in plants, such as stem elongation, is mainly growth by cell enlargement, while the formation of roots or fruits rests in the first stages on a great stimulation of cell division, which only later is followed by enlargement. Yet both these processes are controlled by the supply of auxin to the tissues. The direct effect of auxin on the cambium is also stimulation of cell division. Elsewhere, as in lateral buds, auxin, in physiological concentrations, causes complete inhibition of growth. Such a diversity of the ultimate effects of one hormone suggests strongly that the results observed are remote from the initial action, and that this initial action of auxin on the cell is a fundamental one exerted on some process of metabolism. From this hypothetical change in metabolism the visible effects ensue, according to the age and location of the cell or tissue, the supply of water and of both plastic and catalytic materials, and perhaps also the interaction of auxin with other specific substances.

The purpose of this paper is to consider the evidence that auxin brings about growth through causing a change in metabolism. Now it is known in a general way that growth of higher plants is aerobic and does not take place in nitrogen. This was first shown for a specialized growth reaction, namely geotropic curvature, by van Amejden in 1917. More than ten years ago J. Bonner found (1936) that growth of the oat coleoptile is directly dependent on respiration and is inhibited by cyanide to the same extent that respiration is. On this account van Hulssen tried

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² I wish to acknowledge the assistance and contributions (many of them unpublished) of Dr. Walter D. Bonner, Jr., and of several students, past and present, including Dr. Schneider, Dr. Commoner, Dr. Sweeney, and Mr. Christiansen.

to detect an influence of auxin on respiration, using oat coleoptiles, but found no effect. Others also obtained negative results, although subsequently it has been found that there are some conditions under which auxin may produce an increased respiration. The absence of any necessary increase in respiration to accompany the increase in growth rate, however, indicates that growth by cell enlargement does not involve any considerable overall expenditure of energy. This has been shown in another way by the calculations of Goddard (1948) and of Frey-Wyssling (1948) whose figures indicate that the actual energy involved in growth is probably not over one per cent of the total energy available to the cell from respiration.

Cyanide, of course, acts on the terminal oxidase and thus inhibits the respiration of all kinds of metabolites. Its effect, therefore, is not specific. It was thought that a fresh approach might be made through studying the effects on growth of the somewhat more specific dehydrogenase inhibitors. This at once proved to be fruitful and has led to numerous metabolic experiments with growth inhibitors.

THE EXPERIMENTAL MATERIAL

At this point mention should be made of the experimental objects often used in growth studies. The requirement for strictly uniform plants available in large numbers makes it essential to use seedlings, and, because light influences the production of auxin and also causes curvature and other complications, the seedlings are almost invariably grown in the dark, and worked on only in red light. Of such dark-grown (etiolated) seedlings the most widely used is the oat, the coleoptile of which completes its growth in about five days at 25° C., and in which all cell division ceases after the first 10 mm. is reached. This provides an ideal experimental object, in which the growth involves only cell elongation. Sections cut from such coleoptiles grow well in simple auxin solutions, and much better if sucrose is added. The optimum concentrations are about 1 per cent sucrose and 1 to 5 mg. per liter of indole-acetic acid ($= 0.6$ to 3.0×10^{-5} M). Another very satisfactory etiolated seedling is that of the pea; we use sections cut from the apex of the third internode. The growth of these sections is small (about 50 per cent of their initial length) but very reproducible; it is not appreciably affected by the addition of sugar, and hence this material is convenient for chemical studies. The same internodes when slit lengthwise give a large curvature in auxin solutions which has been extensively used in assaying synthetic substances for their auxin activity; this response has the advantage of not being nearly so limited in its applicability as that of the well known "Avena test" of Went, in which the auxin is applied in agar to one side of the decapitated coleoptile.

The growth of seedling sections in pure auxin solution—with sucrose added in the case of the oat coleoptile—is of course a highly limited growth process. Shorn of the complications due to cell division, mineral nutrition, light and nitrogen supply, these sections represent about the simplest system which can still be regarded as growing. The detailed analysis of such a simplified system should, however, be the first step towards an understanding of the whole complex of growth reactions which takes place under natural conditions.

The growth of coleoptile sections is highly aerobic. Even submergence beneath 1 mm. depth of solution retards it about 50 per cent; aeration of such lightly submerged sections restores the rate to its full value (Thimann and Bonner, 1948). In most of our experiments the sections are arranged so as just to break surface.

GROWTH INHIBITION BY IODOACETATE

The growth is reduced or prevented by inhibitors of dehydrogenases. The effect of iodoacetate was studied in detail by Commoner and Thimann (1941), who showed that growth of coleoptile sections is strongly inhibited by this substance. However, concentrations of the inhibitor which reduce growth practically to zero have only a very small effect on respiration. (The data on respiration are in excellent agreement with the later measurements of J. Bonner, 1948). Here again it follows that of the total energy released by respiration only a few per cent can be needed for growth, for otherwise the complete inhibition of growth could be achieved only with a substantial inhibition of respiration. This confirms the experiments and calculations mentioned above; most of the energy of metabolism evidently goes for maintenance. However, it is not excluded that growth might involve an appreciable fraction of the respiration, but that when this process is inhibited another type of respiration might take its place, so that the total oxygen consumption would show little change. We shall return to this important consideration below. It is also necessary to point out that in the pea stem the relations are not quite the same, for here auxin does produce a slight increase of oxygen consumption, and growth inhibition is accompanied by a definite respiration decrease (see below).

An interesting and important effect was observed with iodoacetate; the sensitivity to this inhibitor varies with the age of the coleoptile. This is shown in Figure 1. Young coleoptiles show an incomplete inhibition, as well as a marked

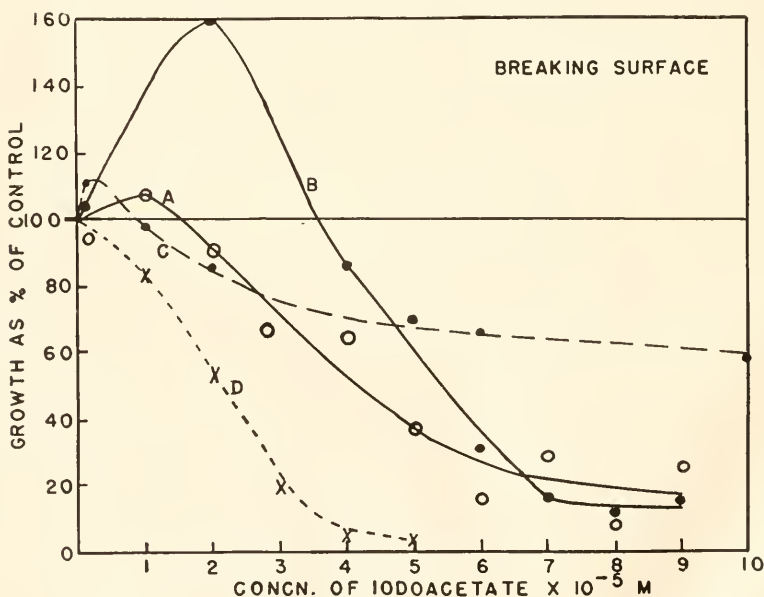


FIGURE 1.³ The total growth, after 48 hours, as per cent of that of the controls, plotted as a function of iodoacetate concentration. Curve A, sections from 74 hr. coleoptiles; curve B, from 64-66 hr. coleoptiles; curve C, from 54-56 hr. coleoptiles; and curve D, from 96 hr. coleoptiles. All solutions contained 1 mg. indole-acetic acid and 10 grams sucrose per liter.

³ Figures 1, 2, 3, and 5 are from Thimann and Bonner (1948 and 1949).

acceleration at low inhibitor concentrations; with the oldest coleoptiles, on the other hand, the concentrations necessary for threshold and for 50 per cent inhibition are much lower, and the maximum inhibition is very high. Two explanations are possible for this effect:

(a) the amount of enzyme with which the iodoacetate has to combine decreases with increasing age;

(b) the young plant contains substances which oppose the inhibition and which decrease in amount with increasing age.

Evidence for the latter view will be presented below. First, however, it will be convenient to consider in more detail the nature of the enzyme system.

SULFHYDRYL NATURE OF THE "GROWTH ENZYME"

It is known that iodoacetate (or iodoacetamide, which behaves similarly) reacts with sulfhydryl groups, although as Michaelis and Schubert have shown (1934) it is not strictly specific for these. Since the enzyme system which controls growth is of great importance, it seemed worth while to determine definitely whether it is of sulfhydryl nature or not. The action of a number of other inhibitors has given clear-cut evidence on this (Thinmann and Bonner, 1949).

Arsenite and the organic arsenical mapharsen inhibit growth strongly. The effective concentrations are lower than for iodoacetate. The extent of inhibition by arsenite, unlike that by iodoacetate, does not vary with the age of the coleoptile (Fig. 2). The growth of pea stems is also inhibited by arsenite, showing exactly

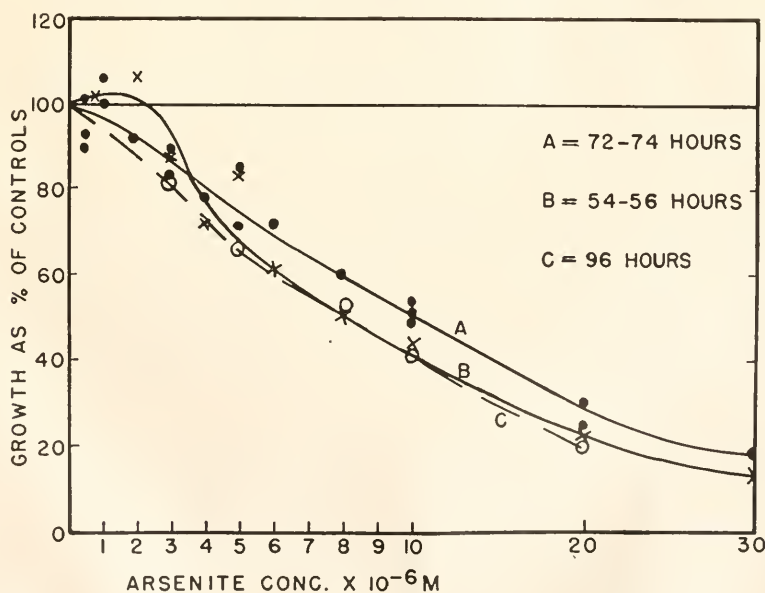


FIGURE 2. Data similar to Figure 1 but for arsenite. Sections from the three ages of coleoptiles show no significant difference in sensitivity to the inhibitor.

the relationship with arsenite concentration to be expected of a titration curve (Fig. 3). Such inhibitions can, of course, be regarded as titration of the enzyme with the inhibitor. The "titration curves" of iodoacetate are complicated by the promotion of growth at low iodoacetate concentrations; arsenite does not produce this effect.

A more specific sulfhydryl reagent is parachloromercuribenzoate, introduced by Hellerman et al. (1943). This also inhibits growth and, like arsenite, the effective concentrations do not vary appreciably with age. Again the growth and slit stem curvatures of peas are also inhibited by this reagent.

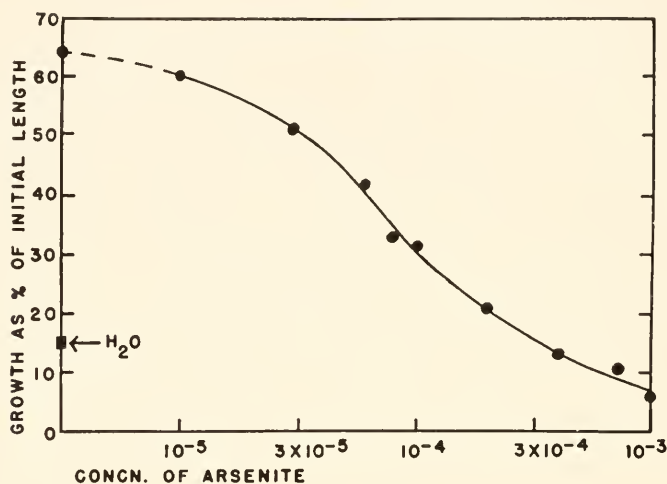


FIGURE 3. Growth, after 24 hrs., of 20 mm. sections of the uppermost internode of 7-day-old etiolated pea stems. All solutions contained indole-acetic acid 10 mg. per liter and arsenite as shown, but no sucrose. Growth of controls in water is shown at left.

Finally the phenyl-mercuric salts inhibit growth strongly. The concentration for 50 per cent growth inhibition of coleoptile sections is very low, about 7×10^{-6} M. However, there is reason to believe that these substances are not so specific as those above, since they definitely inhibit respiration. At concentrations which produce 50 per cent inhibition of growth, arsenite, iodoacetate and parachloromercuribenzoate exert no detectable effect on coleoptile respiration, as shown in Figure 4. The phenyl-mercuric salts thus inhibit somewhat in the same way as cyanide.

Taking the data together it is clear that the growth-controlling enzyme is of sulfhydryl composition. This conclusion holds far beyond the higher plants on which this work was done, for long ago Hammett, Voegtlin, Chalkley and others adduced evidence for the importance of the SH-group in the growth of invertebrates, and recently, Ryan, Tatum and Giese (1944) showed that the growth of *Neurospora* is inhibited by iodoacetate in a manner both quantitatively and qualitatively similar to that of coleoptile sections. This clearly is an aspect of General Physiology.

THE ROLE OF ORGANIC ACIDS IN GROWTH

Some years ago Commoner and Thimann (1941) found that the inhibition by iodoacetate is prevented by malate, succinate, fumarate and pyruvate. More recently we have confirmed this and refined the technique of such experiments. Isocitrate has a similar effect; so also, unexpectedly, do maleate and malonate. The growth system behaves in this respect like succinic dehydrogenase, which has been shown by Hopkins, Morgan and Lutwak-Mann (1938) to be "protected" against iodoacetate by these acids. However, while maleic and malonic acids bring the growth rate in presence of iodoacetate back to normal, they have no further effect; malate, succinate, fumarate and isocitrate bring the growth rate considerably above normal (Fig. 5). In other words these acids accelerate growth. There are thus two phenomena to be distinguished: protection against iodoacetate, and promotion of growth. Maleate and malonate, which protect the SH group against iodoacetate, do not promote growth, and indeed in higher concentrations (0.05 M) actually inhibit it. Growth promotion is limited to the acids mentioned above, with the

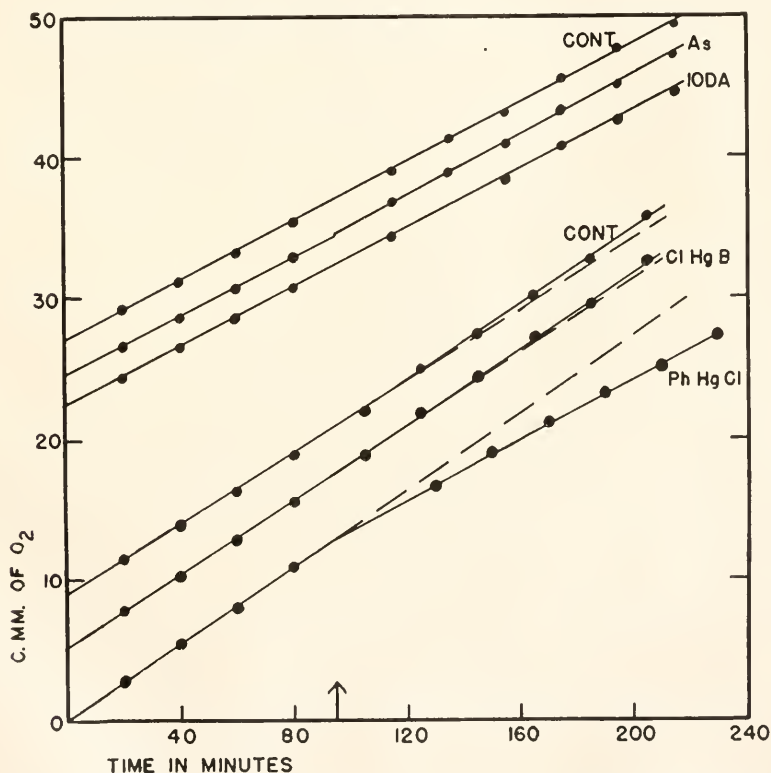


FIGURE 4. The respiration of sections from 72-hour-old coleoptiles in indole-acetic acid 1 mg., sucrose 10 gms., per liter. Two experiments are shown; the uppermost curve being the controls in each case. The inhibitors were added at the arrow, in concentrations which cause 50 per cent decrease in growth. As: arsenite 1.10^{-5} M As_2O_3 . IODA: 5.10^{-5} M iodoacetamide. ClHgB: 4.10^{-5} M parachloromercuribenzoate. PhHgCl: 1.10^{-5} M phenyl-mercuric chloride.

addition of pyruvate (which is very active when pure), of citrate for young plants only, and of acetate.

These acids active in growth are those of the Krebs cycle. The importance of this cycle in respiration is well known, and recently J. Bonner (1948) has made it probable that the same or a similar cycle occurs in the respiration of coleoptile tissue. The experiments above, and several others, make it clear that malate and other acids of the cycle actually participate in the normal growth process.

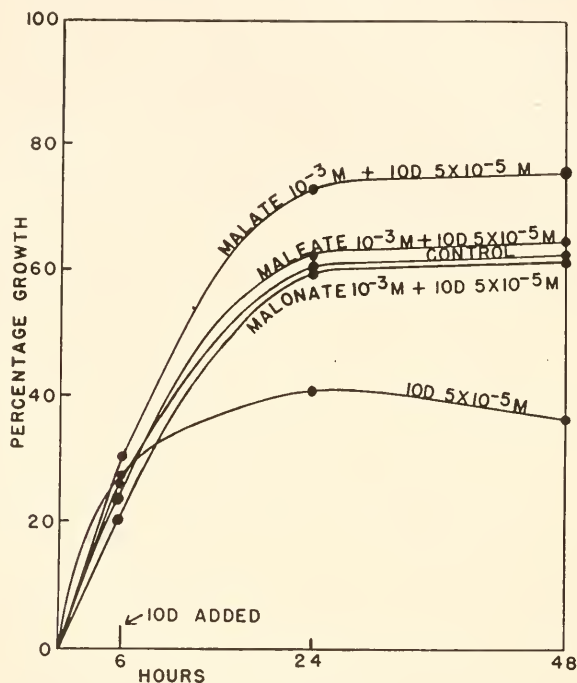


FIGURE 5. The effects of malonate, maleate and malate in preventing the iodoacetate inhibition of growth. Sections cut from 66-hour-old coleoptiles, breaking surface, in solutions containing 1 mg. indole-acetic acid and 10 grams sucrose per liter. The iodoacetate was added 7 hrs. after placing the sections in organic acids. While all three acids act against iodoacetate, only the malate increases growth above that of the controls.

For one thing, treatment with malate makes possible a direct demonstration that auxin does influence respiration in the coleoptile. For if coleoptile sections, which show no increase in respiration when auxin is added, are first soaked for some hours in malate or fumarate, then the addition of auxin at once increases their respiration rate. This means, of course, that malate (etc.) is required for auxin to exert its full effect, and that when this effect is exerted on growth it is by way of a respiratory system.

The role of the organic acids in facilitating the action of auxin also makes possible an explanation of the effect of age on growth. Some years ago, Mrs. Sweeney and I (1942) made a study of the effect of auxin on the rate of protoplasmic streaming in the epidermal cells of the coleoptile. The usual effect of

physiological concentrations of indole-acetic acid or other auxins is to accelerate the rate of streaming; the process requires both oxygen and sugar, and from its dependence on auxin concentration and other features, we deduced that it is closely related to the promotion of growth. Now when the coleoptiles are very old (120 hours) this acceleration of streaming no longer occurs, but it was found that if the old coleoptiles are soaked in malate for some hours, the acceleration is reinstated. This would suggest that in old coleoptiles the concentration of malate has greatly decreased. *A priori* this would seem unlikely since it is known from experiments on the excystment of protozoa that in fully grown grasses the opposite is the case—organic acids increase markedly with increasing age. However, W. Bonner has recently shown by direct microanalysis that the amount of malate and other organic acids in the coleoptile does decrease with increasing age (Table I).

TABLE I
Organic acids of Avena coleoptiles and pea stems
All figures per gram dry wt.

AVENA SECTIONS	TOTAL ETHER-SOLUBLE ACIDS micro-equivalents	CITRIC mg.	MALIC mg.
54 hours	432	1.44	13.7
72 hours	329	1.05	10.2
96 hours	284	1.02	5.7
PISUM STEMS	616	1.29	13.7

Another interesting point shown in the table is that the pea stems contain more organic acids than any age of coleoptile. Now the pea stems do not show increased growth with malate. Furthermore, they differ from the coleoptiles in another important respect: the addition of auxin causes a direct increase in respiration rate. This increase is about 15 per cent and is maintained for 24 hours or throughout the whole period during which growth of the sections takes place. In other words, the pea stems behave like coleoptiles pretreated with malate. The correlation between this behavior and their content of malate and other acids shows how the age effect in coleoptiles is to be interpreted; the increasing sensitivity to iodoacetate with increasing age is due to a decreasing content of the organic acids, which "protect" against iodoacetate. Correspondingly, aging causes no increase in sensitivity to arsenite or parachloromercuribenzoate, because the organic acids do not protect against these reagents.

There are other substances whose decreasing concentrations play a role in growth. Preliminary determinations of the keto-acids indicate that they behave in a similar way. J. Bonner (private communication) has recently shown that arginine also plays a role in growth of coleoptiles, which can be accelerated by adding arginine and inhibited by adding the related substance canavanine. In our experiments the promotion of growth by arginine is limited to older coleoptiles, so that the amount of this substance appears to decrease with age, like the organic acids. Doubtless still other materials behave similarly. However, to be able to

explain even one aspect of aging in terms of a decrease in concentration of known substances in the plant is a definite step forward.

Why should the amount of these substrates decrease with age? To some extent the reason might be merely that the amount contributed by the seed is limited, so that the substances are essentially diluted by the increasing volume of the seedling. But in the case of some of the organic acids there is evidence, which will not be presented here, that they are actually used up in the growth process, and when growth is inhibited, the rate of their disappearance slows down. This brings us to the last part of the subject, namely the phenomena occurring during inhibition.

METABOLIC CHANGES ASSOCIATED WITH GROWTH INHIBITORS

Since the pea stem grows very well in auxin solution without sucrose, and shows little increase in growth when sugar is added, it seemed to us excellent material for a study of the fate of carbohydrates during growth and inhibition. Several conclusions can be drawn from this work. It appears, first, that the amount of reducing sugar which disappears during growth is exactly the same in auxin as in water. When it is considered that the growth in auxin is three or four times that in water, and that the R.Q. in both cases is close to 1, this result is surprising. Changes in non-reducing sugars are small and only minute amounts of metabolizable polysaccharides are present. It follows that the consumption of sugar in growth, both as a metabolic substrate and as a constituent of cell-walls, is negligibly small.

Secondly, when growth is inhibited, reducing sugars do not pile up as might be expected, but instead their amount decreases. With 50 per cent growth inhibition by iodoacetate, the reducing sugar decreases about 25 per cent more than when growing without inhibitor. The same effect is shown by other inhibitors. Typical results are shown in Table II (Christiansen et al, 1949). Fluoride has not been mentioned above as an inhibitor; its effect in lowering the reducing sugar is somewhat greater than that of the sulphydryl-combining inhibitors. This, however, may be due to the fact that it slightly increases respiration after some hours, instead of decreasing it as do the others.

Naturally the fate of the reducing sugar which disappears when growth is inhibited is of the greatest interest. It is not respired away, since respiration is (except with fluoride) decreased. It is not converted to detectable amounts of starch, and chemical analyses show that it is not deposited as cellulose or any other wall materials. It is not converted to phosphate esters either, since the amounts of these, both in inhibited and uninhibited sections, are very small in comparison to the amounts of sugar involved.

In brief it appears that, though the effect is qualitatively the same with different inhibitors, the ultimate fate of the sugar differs in each case. In arsenite the sugar is converted to neutral ether-soluble material, i.e. fats. In fluoride the same conversion occurs but some of the material is respired away, while in iodoacetate, surprisingly enough, it is excreted into the solution in the form of organic acids, or, more strictly, a quantity of organic acid equal in weight to the sugar which has disappeared is excreted into the solution. It should be noted that growing tissues normally accumulate solutes vigorously from solution so that an excretion (or exosmosis) in quantity is most unusual. The nature of this stem exudate is now under investigation; it contains some fructose and 15 per cent of asparagine.

In general the experiments show that inhibitors not only inhibit one process (which leads to growth), but they also promote another, which consumes sugars. In this respect the phenomena are suggestively similar to the "uncoupling" of phosphorylation by dinitrophenol (Loomis and Lipmann, 1948) which also leads to increased disappearance of sugar. Indeed dinitrophenol does inhibit growth, though we have not yet studied it in detail. Space does not permit the detailed presentation of data on other inhibitors but it may be mentioned (a) that fluoroacetate, which specifically inhibits acetate metabolism, causes a partial growth inhibition which can be reversed by adding acetate, and (b) that poisoning the enzyme which decarboxylates pyruvic acid does not inhibit, but actually promotes growth.

TABLE II

*Growth, respiration and reducing sugar of 20 mm. sections
of etiolated pea stems after 24 hrs. in solution*

	GROWTH as per cent elongation	REDUCING SUGAR as per cent of fresh wt.	RESPIRATION as Q_{O_2} (after 12 hrs.)	PER CENT DECREASE		
				of growth in length	of reducing sugar	of respiration
Initial	—	1.12	—	—	—	—
In water	20.0	0.83	3.34	—	—	—
In auxin (1 mg./l.)	50.9	0.86	3.82	0	0	—
In auxin plus: Iodoacetate 6×10^{-4} M	25.6	0.64	3.32	50	26	13
1×10^{-3} M	18.2	0.54	—	64	37	—
Arsenite 1×10^{-4} M	26.5	0.71	2.89	48	17	24
1×10^{-3} M	0	0.51	—	100	41	—
Fluoride 5×10^{-3} M	25.3	0.48	4.47	50	44	17
1×10^{-2} M	17.8	0.42	—	65	51	—

The relation between respiration and growth is thus not a simple one. The process mediated by the SH-enzyme appears to be a major limiting factor. It either consumes only a small part of the total oxygen and sugar used, or else it is readily replaced by an equivalent reaction not causing growth, and it probably is a step in the oxidation of pyruvic acid via the Krebs cycle or some modification of it. The SH-enzyme is almost certainly one of the dehydrogenases of this cycle. It may be noted in passing that attempts to demonstrate an iodoacetate-inhibited enzyme of this type in coleoptile brei, by Berger and Avery (1944), failed, but this would be expected from the data presented above, because the test has to be

made in presence of malate or other dicarboxylic acid, and these very substrates protect the enzyme fully against the inhibition by iodoacetate. It also appears that the simple decarboxylation of pyruvic acid does not lead to growth, but its oxidative decarboxylation and the resulting metabolism of acetate does do so. The inhibition of growth in presence of auxin is not the same as the mere absence of growth in sections not supplied with auxin; it is a positive process and leads to the diversion of sugar metabolism into other pathways. The action of an inhibitor is that of a switch, diverting the stream of traffic into a direction which does not lead to growth. Correspondingly the action of auxin itself may also be that of a switch, causing the metabolism of carbohydrate, or perhaps more specifically that of pyruvate, to go via the growth-promoting system instead of by another. It may be, therefore, that the apparently small fraction of metabolism involved in growth is a misleading phenomenon and that the true picture is rather one of alternative routes involving approximately equivalent amounts of respiration. However, such considerations are at present only speculative.

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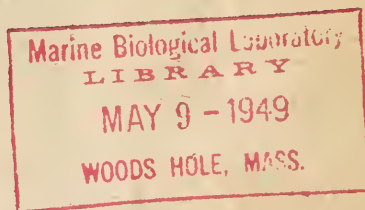
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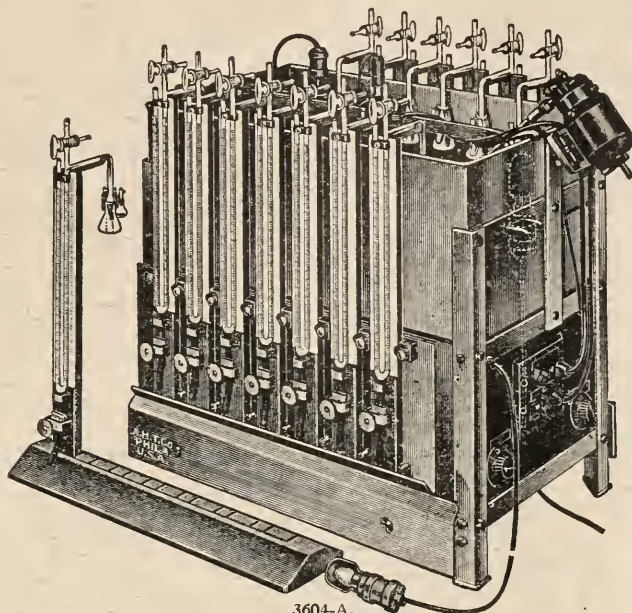
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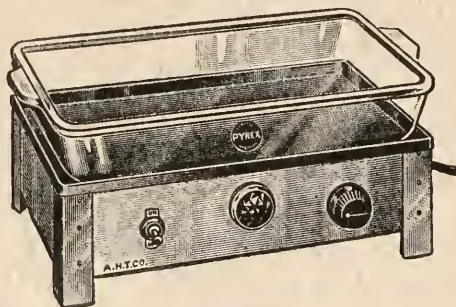
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